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(71) Applicants: **VIB VZW** [BE/BE]; Rijvisschestraat 120, 9052 Gent (BE). **KATHOLIEKE UNIVERSITEIT LEUVEN, K.U.LEUVEN R&D** [BE/BE]; Waaistraat 6 - bus 5105, 3000 Leuven (BE).

(72) Inventors: **MAZZONE, Massimiliano**; Ketelmakerij 19, 3010 Kessel-Lo (BE). **OLIVEIRA, Ana**; Arnould Nobelstraat, 28, 0302, 3000 Leuven (BE). **CELUS, Ward**; Lakerweg 2, 3640 Kinrooi (BE).

(74) Common Representative: **VIB VZW**; Rijvisschestraat 120, 9052 Gent (BE).

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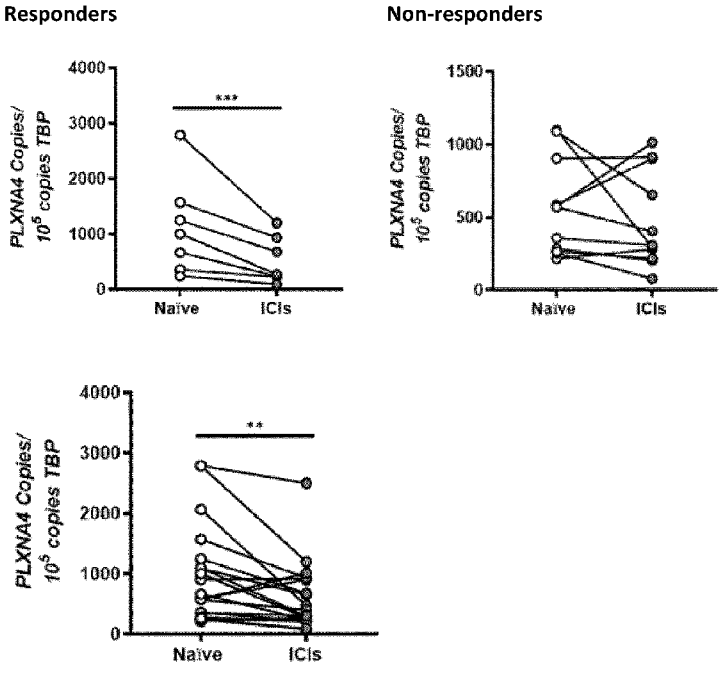

FIGURE 1

(57) Abstract: The invention relates to the field of biomarkers useful in monitoring anti-tumor therapy response, in particular response to immune checkpoint inhibitors. In particular, the biomarker identified herein is a plexin of the A-class, in particular plexin A4. More in particular expression of a plexin of the A-class in circulating T-cells, in particular circulating CTLs, is an early non-invasive biomarker for response to anti-tumor therapy. Methods of using this biomarker as well as kits relying on this biomarker are part of the invention.

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Biomarker for anti-tumor therapy

FIELD OF THE INVENTION

The invention relates to the field of biomarkers useful in monitoring anti-tumor therapy response, in particular response to immune checkpoint inhibitors. In particular, the biomarker identified herein is a plexin of the A-class, in particular plexin A4. More in particular expression of a plexin of the A-class in circulating T-cells, in particular circulating CTLs, is an early non-invasive biomarker for response to anti-tumor therapy. Methods of using this biomarker as well as kits relying on this biomarker are part of the invention.

BACKGROUND OF THE INVENTION

Plexins are large transmembrane glycoproteins that function as the receptors/ligands for the axon guidance proteins named semaphorins (Perala et al. 2012, Differentiation 83:77-91; Battistini & Tamagnone 2016, Cell Mol Life Sci 73:1609-1622). Accordingly, for several years, the research on this topic was focused on the nervous system, where they play a bifunctional role, having the capacity to exert both repulsive and attractive effects (He et al. 2002, Sci STKE 2002(119):re1). These chemoattractant properties together with the discovery of their role in immune responses in both physiological and pathological conditions (Kumanogoh & Kikutani 2013, Nat Rev Immunol 13:802-814; Roney et al. 2013, Protein Cell 4:17-26) led to the study of semaphorin signaling in the tumor microenvironment (TME; Capparuccia et al. 2009, J Cell Sci 122:1723-1736). It was already demonstrated that blocking of Sema3A signaling plays a key role in restoring anti-tumor immunity by impeding tumor associated macrophages (TAMs) to enter hypoxic niches (Casazza et al. 2013, Cancer Cell 24:695-709). Additionally, it was shown that Sema4A signaling promotes Treg cell stability in the TME (Delgoffe et al. 2013, Nature 501:252-256). Within the class A plexins, four members are currently known: A1, A2, A3, and A4.

Plexin-A1 expression levels were found strongly correlated with glioma grade. Inhibiting plexin-A1 downstream signaling in glioma cells with a synthetic transmembrane targeting peptide MTP-Plexin-A1 peptide was shown to reduce glioblastoma tumor growth (Angelopoulou & Piperi 2018, Cancer Lett 414:81-87; and references cited therein). Plexin-A1 may together with Sema6B be involved in tumor angiogenesis (Lu et al. 2016, Oncol Lett 12:3967-3974).

The role of Plexin A2 (PlxnA2) in the immune system is unknown. The ligands of PlxnA2 appear to overlap with those of PlxnA4, and PlxnA2 was described as a repulsive guidance molecule in the central nervous system (Suto et al. 2007, Neuron 53:535-547; Shim et al. 2012, Mol Cell Neurosci 50:193-200).

Plexin A3 is a high-affinity cooperative receptor of NRP2 and appears involved in neuronal cell migration and axon guidance (Waimey et al. 2008, Dev Biol 448-458; Yaron et al. 2005, Neuron 45: 513-523). Plexin A3 was reported to be expressed on oligodendrocyte precursor cells and to be involved in their migration (Xiang et al. 2012, Neurosci Lett 530:127-132).

Plexin A4 (PlxnA4) is a member of class A plexins (Fujisawa 2004, J Neurobiol 59:24-33) that acts as the interactor of class 6 semaphorins (Battistini et al. 2013, Cell Mol Life Sci 73:1609-1622). Together with neuropilin 1 (Nrp1), it can also function as a co-receptor for class 3 semaphorins (Fujisawa et al. 2004, J Neurobiol 59:24-33). In the central nervous system, PlxnA4 was found to be a potent mediator of axon-repulsive activities by the direct binding to class 6 transmembrane semaphorins, Sema6A and Sema6B (Suto et al. 2005, J Neurosci 25:3628-3637; Tawarayama et al. 2010, J Neurosci 30:7049-7060). Nevertheless, in the immune system, it has different functions. On one hand, PlxnA4 seems to have a positive role in Toll-like receptor (TLR)-mediated signaling and macrophage cytokine production, as *Plxna4*-deficient mice have attenuated TLR-mediated inflammation, including septic shock (Wen et al. 2010, J Exp Med 207:2943-2957). On the other hand, the same mice showed enhanced T cell priming and exacerbated disease in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Yamamoto et al. 2008, Int Immunol 20:413-420). International Patent Publications WO 2001/014420, WO 2012/114339 and WO 2015/037009 are related to Plexin-A4. WO 2001/014420 discloses plexin-A4 as novel member of the plexin family; WO 2012/114339 focuses on molecules binding to type A plexins and inhibiting proliferative signals trough the type A plexin receptor without interfering with binding of the type A plexin to neuropilin or semaphorin 6A. WO 2015/037009 discloses antibodies binding to Plexin-A4. The application of plexin A4 is a biomarker for Alzheimer's is disclosed in US20160186263A1.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to methods for monitoring response of a subject having a tumor to anti-tumor therapy, such methods comprising quantifying an amount of biomarker which is a plexin of class A in a first and a second fluid sample;

wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy to the subject, and said first fluid sample is obtained from the subject before or at administration of the at least one dose of the anti-tumor therapy to the subject or said first fluid sample is a control sample; and

wherein a reduction of the amount of the biomarker in said second fluid sample compared to said first fluid sample is predictive for the subject to be responsive to said anti-tumor therapy; and wherein the

absence of a reduction of the amount of the biomarker in the second fluid sample compared to the first fluid sample is predictive for the subject not to be responsive to said anti-tumor therapy.

Herein, said biomarker may be human plexin A4 (plxnA4) or human plexin A2 (plxnA2); and the fluid sample may be selected from fluids such as blood, urine, saliva, or a fraction thereof.

In particular, the above methods may further comprise a step of enriching T-cells from said fluid sample to obtain an enriched sample and quantifying the amount of the biomarker in said enriched sample.

In any of the above methods, the quantification of the amount of the biomarker may comprise quantifying the amount of a nucleic acid of said biomarker, preferably the amount of RNA of said biomarker.

In any of the above methods, said tumor may be a solid tumor or an inflamed tumor. In particular, the tumor may be melanoma.

In any of the above methods, the anti-tumor therapy may be comprising anti-tumor immunotherapy, such as comprising immune checkpoint inhibitory therapy, such as comprising administration of an inhibitor of PD-1, PD-L1, or CTLA4.

In any of the above methods, the second fluid sample is obtained from the subject two to four weeks after administration of a dose of the anti-tumor therapy.

Another aspect of the invention relates to plexin class A antagonists for use in the treatment of a subject having a tumor, wherein the subject has been indicated to be not responsive to an anti-tumor therapy not comprising a plexin class A antagonist using any of above methods. Herein, the anti-tumor therapy not comprising a plexin class A antagonist may be replaced or combined with a second anti-tumor therapy. In particular, such second anti-tumor therapy may be comprising administration of a plexin class A antagonist.

The invention further relates to kits comprising at least one oligonucleotide to quantify the amount of a plexin of class A biomarker for use in any of the above methods.

LEGENDS TO THE FIGURES

Figure 1. *Plxna4* expression in circulating CD8$^+$ T cells of melanoma patients responding or not responding to immune checkpoint inhibitor (ICI) therapy. Expression of *Plxna4* in isolated CD8$^+$ T cells before (naïve) and after a single dose of ICI-therapy (ICIs) and this in patients responding to ICI-therapy (top left panel) or not responding to ICI-therapy (top right panel) according to the RECIST criteria. In the bottom left panel, expression of *Plxna4* as determined for all patients is depicted; this data set includes patients for which a clinical determination of response or non-response is not yet available.

Figure 2. PlxnA4 loss in CD8+ T-cells increases their migratory capacity. (A) *Plxna4* expression in CD8+ T-cells sorted from subcutaneous LLC tumor-bearing WT mice. (B) *Plxna4* expression in sorted CD8+ T-

cells before and after *ex-vivo* activation with CD3/CD28 dynabeads for 4 days; (C) Migration of WT and *Plxna4*-KO CD8+ T-cells towards CCL21 and CCL19; (D-F) Homing of WT and *Plxna4* KO CD8+ T-cells to the lymph nodes assessed by FACS (D), quantification by histology (E) and a representative micrograph (F); (G-H) FACS analysis of CD8+ T-cells in the draining LNs of WT and *Plxna4* KO mice bearing subcutaneous LLC tumors (G), or in chimeric WT→WT and *Plxna4* KO→WT mice bearing orthotopic E0771 tumors (H) ; (I) Migration of WT and *Plxna4*-KO CD8+ T-cells towards CXCL9 and CXCL10. For the *in vivo* experiments, n= 4 (A) and n= 5-6 (D-H). (J) Homing of naïve WT and *Plxna4* KO $CD8^+$ T cells to the lymph nodes of WT mice treated with vehicle or FTY720 (fingolimod). (K-L) Tumor homing of activated WT and *Plxna4* KO OT-I T cells to LLC-OVA tumor-bearing mice (J) or B16-F10-OVA tumor-bearing mice (K) assessed by flow cytometry 24 hours (J-K) and 48 hours (K) after T cell injection. *In vitro* results (B-C, I) are representative of at least two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ versus LNs (A), naïve CD8+ T-cells (B), WT (C-G), WT→WT control (H), or WT OT-I T-cells (K,L). Scale bar: 100 μm. All graphs show mean ± SEM.

Figure 3. PlxnA4 KO CD8+ T-cells have increased proliferation index and present more effective anti-tumor responses. (A-C) *Ex-vivo* proliferation of WT and *Plxna4* KO Violet Cell Tracer-labelled splenocytes upon CD3/CD28 activation showing percentage of CD8+ T-cells (A), proliferation index (B) and a representative histogram of Violet Cell Tracer fluorescence intensity, gated on $CD8^+$ cells, after 4 days in culture (C). (D) FACS analysis of CD69 activation marker in the draining LNs of WT and *Plxna4* KO subcutaneous LLC tumor-bearing mice; For the *in vivo* experiments, n= 5-6 (D). *In vitro* results (A-C) are representative of at least two independent experiments. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ versus WT (A-C) or WT→WT control (D). (E) FACS analysis of B16-F10-OVA tumors 24 hours after intratumoral injection of WT and *Plxna4* KO OT-I T cells. All graphs show mean ± SEM. Dashed lines represent FMO controls. MFI, Median Fluorescent Intensity; FMO, Fluorescence Minus One; ns, not-significant versus WT control. (F) Subcutaneous LLC tumor weight (B) in mice with a full deletion of *Plxna4* (KO in short) and control littermates (WT in short); n= 8 (B, two independent experiments pooled). **$p < 0.01$, and ****$p < 0.0001$ versus WT. ns, not-significant versus WT. Graphs show mean ± SEM.

Figure 4. Adoptive T cell transfer (ACT) of WT and KO OT-I $CD8^+$ T cells in LLC-OVA or B16-F10-OVA tumor bearing mice. (A) Tumor growth model in subcutaneous LLC-OVA tumor bearing mice, with ACT at day 5 (A) after tumor inoculation. (B) Tumor growth model in subcutaneous B16-F10-OVA tumor bearing mice, with ACT at day 13 (B) after tumor inoculation. (A-B) Comparison of WT and KO OT-I $CD8^+$ T cells and PBS as control. (C) Survival effect (Kaplan-Meier overall survival curves) of ATC with WT and KO OT-I $CD8^+$ T cells in subcutaneous B16-F10-OVA tumor bearing mice.

(D) weight and (E) representative images of end-stage B16-F10-OVA tumor tumors; (E):scale bar = 2 cm.

(F) FACS analysis of intratumoral OT-I T cells in B16-F10-OVA tumors isolated 4 days after ACT.

Groups n= 5-6 . # p<0.0001 versus PBS control, **p < 0.01. All graphs show mean ± SEM.

Figure 5. PlexinA2-specific deletion in CD8+ T cells increases anti-tumor immunity. (A-B) PlexinA2 mRNA expression in CD8+ T-cells in tissues of normal and tumor-bearing mice. (A) PlexinA2 mRNA expression is high in FACS sorted CD8+ T cells from blood as compared to LNs and spleen of healthy WT mice. (B) PlexinA2 is highly expressed in FACS sorted CD8+ T cells from blood while expressed at a lower level in sorted CD8+ T cells from lymph node (LN), tumor-draining LNs, spleen and primary tumor of subcutaneous LLC tumor-bearing WT mice. (C-D) Effect of CD8-positive T-cell-specific deletion of PlxnA2 on tumor volume (C) and tumor weight (D) in a subcutaneous MC38 colon adenocarcinoma tumor model. (E-F) Effect of CD8-positive T-cell-specific deletion of PlxnA2 on tumor volume (E) and tumor weight (F) in a orthotopic E0771 breast tumor model (G-H) Tumor-infiltration of CD8+ T cells in PlxA2 lox/lox and PlxnA2 +/+ mice containing E0771 tumors (percentage of live cells). FACS analysis of E0771 tumors (sacrifice at day 16) with a specific deletion of CD8+ T cells showed increased number of blood circulating CD8+ T-cells (H) and more CD8+ T-cell infiltration in the primary tumor (G) as compared to their littermate controls.

Figure 6. Type A plexin expression in circulating CD8+ T cells obtained from healthy individuals or obtained from melanoma patients. Top left: expression of plexin A4 (*PLXNA4*); top right: expression of plexin A1 (*PLXNA1*); bottom left: expression of plexin A2 (*PLXNA2*); bottom right: expression of plexin A3 (*PLXNA3*).

Figure 7. Expression of plexin A4 (*PLXNA4*) in circulating CD4+ T cells (left panel) and in circulating monocytes (right panel) obtained from healthy individuals (left bars) and from melanoma patients (circulating CD4+ T cells; right bar) or from different tumor types (circulating monocytes; right bar).

DETAILED DESCRIPTION OF THE INVENTION

In work leading to the current invention expression of a plexin of the A-class, in particular in circulating T-cells isolated from a subject having cancer or a tumor, was identified as a biomarker for response to anti-tumor therapy. In particular, expression of this non-invasive biomarker is decreasing when the cancer or tumor is responding to the anti-tumor therapy. Furthermore, such decrease in expression of the biomarker can be observed already early on in the anti-tumor therapy, even prior to clinically establishing or clinically confirming a response to the anti-tumor therapy. In particular, the anti-tumor therapy is comprising an immune checkpoint inhibitor (ICI).

Therefore, the invention in one aspect relates to methods for monitoring the response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the

response of a subject having a tumor to an anti-tumor therapy, such methods comprising one or more of the following steps:

- obtaining a first fluid sample and a second fluid sample from said subject, wherein said first sample is obtained at or before at least one administration of the anti-tumor therapy, and said second sample is obtained after at least one administration of the anti-tumor therapy, after at least one administration step of the anti-tumor therapy, or after administration of at least one dose of the anti-tumor therapy;
- quantifying, determining, assessing, or measuring an amount of biomarker which is a class A plexin in said first and second sample;

wherein a reduction of the amount of the biomarker in said second sample compared to said first sample indicates that the subject is responsive to said anti-tumor therapy, or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of the biomarker in the second (fluid) sample compared to the first (fluid) sample indicates that the subject is not responsive to the anti-tumor therapy, or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Alternatively, the invention in one aspect relates to methods for monitoring the response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such methods comprising (a step of) quantifying, determining, assessing, or measuring an amount of a class A plexin biomarker in a first and a second fluid sample; wherein said first fluid sample is obtained at or before at least one administration of the anti-tumor therapy, and said second fluid sample is obtained after at least one administration of the anti-tumor therapy, after at least one administration step of the anti-tumor therapy, or after administration of at least one dose of the anti-tumor therapy; and wherein a reduction of the amount of the biomarker in said second (fluid) sample compared to said first (fluid) sample indicates that the subject is responsive to said anti-tumor therapy, or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of the biomarker in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy, or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Herein said first fluid sample or first sample is obtained before or at (the time of) at least one administration of (at least one dose of) the anti-tumor therapy, and said second fluid sample or second sample is obtained after at least one administration or after administration of at least one dose of the anti-tumor therapy. This implies that the first sample can be obtained prior to or at administration of

dose N (i.e. at a time point at which the administered anti-tumor therapy is not yet effective/has not yet kicked in), and the second sample can then be obtained after administration of dose N or dose N+X.

If the number N herein is 1, then the first sample is obtained before starting the anti-tumor therapy (i.e. prior to administration of the first dose of the anti-tumor therapy); the second sample can then be obtained after administration of the first (N=1; this would be the case for an anti-tumor therapy consisting of a single dose administration), or can be obtained after administration of the second (N=1, X=1), third (N=1, X=2), etc. dose of the anti-tumor therapy if the anti-tumor therapy is comprising administration of multiple doses.

If the number N herein is e.g. 2, then the first sample can be obtained before starting the anti-tumor therapy (i.e. prior to administration of the first dose of the anti-tumor therapy), or at any time between the time point before starting the anti-tumor therapy and administration of the second dose, e.g. before or after administration of the first dose, just prior to administration of the second dose, etc. The second sample then is obtained after administering the second dose.

In one embodiment, the first (fluid) sample is a control or reference sample (see further).

Therefore, the invention in a further aspect relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such methods comprising one or more of the following steps:

- obtaining a fluid sample from said subject, wherein said (fluid) sample is obtained during anti-tumor therapy;
- quantifying, determining, assessing, or measuring an amount of biomarker which is a class A plexin in said sample;

wherein a reduction of the amount of the biomarker in said (fluid) sample compared to a control or reference sample (or compared to a standard value) indicates that the subject is responsive to said anti-tumor therapy, or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of the biomarker in the (fluid) sample compared to a control or reference sample (or compared to a standard value) indicates that the subject is not responsive to the anti-tumor therapy, or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Alternatively, the invention in another aspect relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such methods comprising (a step of)

quantifying, determining, assessing, or measuring an amount of a class A plexin biomarker in a fluid sample obtained during anti-tumor therapy; and wherein a reduction of the amount of the biomarker in said (fluid) sample compared to a control or reference sample (or compared to a standard value) indicates that the subject is responsive to said anti-tumor therapy, or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of the biomarker in the (fluid) sample compared to a control or reference sample (or compared to a standard value) indicates that the subject is not responsive to the anti-tumor therapy, or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Alternatively, the invention in another aspect relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, the method comprising quantifying, determining, assessing, or measuring the amount of biomarker which is a plexin of class A in a first and a second fluid sample;

wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy and said first fluid sample is obtained from the subject before or at administration of the at least one dose of the anti-tumor therapy or said first fluid sample is a control sample; and

wherein a reduction of the amount of the biomarker in said second fluid sample compared to said first fluid sample indicates that the subject is responsive to said anti-tumor therapy, or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of the biomarker in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy, or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

The control or reference sample referred to in the above methods is a sample of a subject or is a mixture of samples of one or more subjects, the subjects having the tumor of interest in view of the applied anti-tumor therapy. Instead of a control sample, a standard value of expression of an individual class A plexin can be used for purposes of detecting changes in expression of a class A plexin or plexin A-type biomarker compared to such standard value. A standard value of class A plexin expression may for instance be derived or averaged from fluid, cell-, tissue-, or organ samples of a plurality of subjects having the tumor (such collection of 1 or more samples in fact constituting the control sample). Even more in particular, a control or reference sample, or standard value, can be established for each defined tumor stage, such

as in the absence of the anti-tumor therapy. The amount of biomarker in the second fluid sample can then be compared to the one or more of the standard values.

A control or reference sample, or standard value, can also be established for each defined tumor stage in the presence of the anti-tumor therapy, and possibly further subdivided in control samples/reference samples/standard values for responders to anti-tumor therapy, control samples/reference samples/standard values for non-responders to anti-tumor therapy, and/or control samples/reference samples/standard values for partial responders to anti-tumor therapy. The amount of biomarker in the second fluid sample can then be compared to the one or more of the matched standard values (matched in the sense of e.g. stage during anti-tumor therapy) to decide whether the subject from which the second fluid sample was obtained is a responder, a non-responder or a partial responder to the anti-tumor therapy. For example, when the amount of biomarker in the second fluid sample at a certain tumor stage is matching with the control/reference amount of biomarker or within the range of biomarker control/reference amounts established for a responder in that same tumor stage, then it can be concluded that the subject or the subject's tumor is responding to the anti-tumor therapy.

Alternatively, the control sample is a fluid, cell-, tissue-, or organ sample, or one of a series of fluid, cell-, tissue-, or organ samples, of the same subject having the tumor but taken at an earlier time-point compared to the sample to be newly analyzed (detection of changes in class A plexin expression of a later sample compared to an earlier sample of the same subject). Such earlier time or time-point may be before, at, or early after start of any anti-tumor therapy (such as but not limited to immune checkpoint therapy, immunotherapy or immunogenic therapy; "early after" herein is meant a period of time during which the therapy has not yet significantly affected the disease targeted by the therapy), or at any earlier time or time-point after start of any anti-tumor therapy (such as but not limited to immune checkpoint therapy, immunotherapy or immunogenic therapy) but preceding collection of the sample to be newly analyzed.

The invention thus in another alternative aspect relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, the method comprising quantifying, determining, assessing, or measuring the amount of biomarker which is a plexin of class A in a fluid sample obtained from the subject during anti-tumor therapy; and

comparing the amount of the biomarker in said sample with a set of reference values of biomarker amount obtained during anti-tumor therapy wherein the set comprises reference values of biomarker amount corresponding to responders and non-responders to the anti-tumor therapy; and determining from said comparison whether the subject is responding (or not responding) to the anti-tumor therapy.

In particular, the set of reference values of biomarker amount corresponding to responders may be comprising multiple reference values corresponding to multiple time-points during anti-tumor therapy (e.g. linked to administration of dose N or of dose N+X of the anti-tumor therapy); and set of reference values of biomarker amount corresponding to non-responders may be comprising multiple reference values corresponding to multiple time-points during anti-tumor therapy (e.g. linked to administration of dose N or of dose N+X of the anti-tumor therapy).

Decrease of expression of a class A plexin biomarker of the invention thus is relative to an earlier sample, relative to a control or reference sample, or relative to a standard value as described above. Considered as decreased expression of an individual class A plexin biomarker is a decrease in the number of the class A analyte strands (gene expression, see further) or detected class A plexin protein (protein expression), respectively, of 5% or more, of 10% or more, of 15% or more, of 20% or more, or 25% or more, of 30% or more, of 35% or more, of 40% or more, of 45% or more, of 50% or more, or 55% or more, of 60% or more, of 65% or more, of 70% or more, of 75% or more, of 80% or more, of 85% or more, of 90% or more, of 95% or more, of 100%, of up to 10%, up to 20%, of up to 30%, of up to 40%, of up to 50%, of up to 60%, of up to 70%, of up to 80%, of up to 90%, or of up to 100%. Further considered as decreased expression of an individual class A plexin biomarker is a decrease in the number of analyte strands from equal to or higher than the detection limit to below the detection limit.

In referring to genes or proteins herein, no distinction is made in the annotation. Thus, whereas for example the human plexin A1 gene would be referred to as the *PLXNA1* gene, the mRNA as *PLXNA1* mRNA, and the protein as PLXNA1, such distinction is not made hereinabove or hereinafter.

Class A Plexins

Plexins are membrane proteins known as being involved in semaphorin (Sema) signaling, a process that involves co-receptors such as neuropilins (Nrps) as well as receptor tyrosine kinases (RTKs) such as VEGFR2, Met, ErbB2 and off-track (OTK). Semaphorins are involved in regulating morphology and motility of a plethora of cell types. Four classes (A to D) of plexins are known in vertebrates (Alto & Terman 2017, Methods Mol Biol 1493:1-25).

Plexin-A1

Aliases of plexin-A1 provided in GeneCards® include NOV; Semaphorin Receptor NOV; PLXN1; Plexin 1; PLXNA1; and NOVP. The genomic locations for the PLXN1 gene are chr3:126,983,259-127,037,392 (in GRCh38/hg38) and chr3:126,707,437-126,756,235 (in GRCh37/hg19). The GenBank reference PLXN1

mRNA sequence is known under NM_032242.3. Inhibitory RNA products and CRISPR PLXN1 knockout products are available through e.g. Origene.

Plexin-A2

The GeneCards Human Genome Database provides Plexin A2, Semaphorin Receptor OCT, Transmembrane Protein OCT, Plexin 2, PLXN2, OCT, and KIAA0463 as aliases for PLXNA2. The genomic locations for the PLXN2 gene are chr1:208,022,242-208,244,384 (in GRCh38/hg38) and chr1:208,195,587-208,417,665 (in GRCh37/hg19). The GenBank reference PLXNA2 mRNA sequence is known under accession no. NM_025179.4. Inhibitory RNA products and CRISPR PLXN2 knockout products are available through e.g. Origene.

Plexin-A3

Aliases of plexin-A1 provided in GeneCards® include plexin A3; Semaphorin Receptor SEX; XAP-6; Sex Chromosome X Transmembrane Protein Of HGF Receptor Family 3; HSSEXGENE; PLXNA3; and PLXN3. The genomic locations for the PLXN3 gene are chrX:154,458,281-154,477,779 (in GRCh38/hg38) and chrX:153,686,621-153,701,989 (in GRCh37/hg19). The GenBank reference PLXN3 mRNA sequence is known under NM_017514.5. Inhibitory RNA products and CRISPR PLXN1 knockout products are available through e.g. Origene.

Plexin-A4

The GeneCards Human Genome Database provides Plexin A4, PLXNA4A, PLXNA4B, Epididymis Secretory Sperm Binding Protein, FAYV2820, PRO34003, KIAA1550, and PLEXA4 as aliases for PLXNA4. The genomic locations for the PLXN3 gene are chr7:132,123,332-132,648,688 (in GRCh38/hg38) and chr7:131,808,091-132,333,447 (in GRCh37/hg19). The GenBank reference PLXNA4 mRNA sequences are known under accession nos. NM_001105543.1, NM_020911.1, and NM_181775.3. Inhibitory RNA products and CRISPR PLXN1 knockout products are available through e.g. Origene.

Functional plexin-A, such as plexin-A2 or plexin-A4, when referred to herein, is defined as plexin-A that is expressed and to which no “foreign” (in the sense of non-naturally occurring, artificially made, man-made, or any combination thereof) compound such as pharmacological inhibitor is bound or linked, wherein the “foreign” compound is capable of interfering directly (e.g. competing) or indirectly (e.g. by inducing degradation of plexin-A) with the binding of plexin-A with any one of its potential natural binding partners (including for instance, binding to itself in case of homo-(di-)merization, or to one-another in case of hetero-(di-)merization). Functional plexin-A may be exposed on the surface of CD8+ T-cells, or may be stored inside CD8+ T-cells such as stored in a manner allowing quick release to the cell surface.

As such, functional plexin-A can be lacking on and/or in a cell by repressing, inhibiting, or blocking expression of plexin-A, or by binding of a "foreign" compound (as meant hereinabove) to plexin-A. Genetic modification of CD8+ T-cells isolated from a subject is one means of forcing the CD8+ T-cells to lack functional plexin-A. Such genetic modification can be aimed at repressing, reducing, or inhibiting ongoing expression of plexin-A in the isolated (unmodified) CD8+ T-cells, and/or can be aimed at preventing or inhibiting de novo expression of plexin-A, e.g. in case expression of plexin-A is low or non-existing in the isolated (unmodified) CD8+ T-cells.

Shielding (part of the) plexin-A protein exposed on the surface of CD8+ T-cells and/or stored within CD8+ T-cells by means of contacting the CD8+ T-cells with a pharmacological inhibitor of plexin-A is another means of causing CD8+ T-cells to lack functional plexin-A. The said shielding can be envisaged as neutralizing (part of the) plexin-A protein for interaction with other (natural) binding partners. Such pharmacological inhibitors *per se* are known in the art, see e.g. WO 2012/114339 and WO 2015/037009, and alternatives are discussed in more detail hereinafter. In particular, such pharmacological inhibitors bind to plexin-A2 with high specificity and/or, optionally, with high affinity. Plexin-A protein present inside CD8+ T-cells or on the surface of CD8+ T-cells can further be the target of pharmacologic knock-down such as by molecules or agents inducing specific proteolytic degradation of plexin-A protein.

The agent causing CD8+ T-cell to (substantially) lack functional plexin-A or causing neutralization of plexin-A as referred to herein may be part of a larger molecule further comprising a moiety directing the agent to CD8+ T-cells.

Anti-tumor therapy

Anti-tumor therapy in its broadest sense implies administration of an anticancer agent to a subject having cancer. The term anticancer agent is construed herein broadly as any agent which is useful or applicable in the treatment of a tumor or cancer in a subject. Anticancer agents comprise chemotherapeutic agents (usually small molecules) such as alkylating antineoplastic agents, anti-metabolites, anti-microtubule agents, topoisomerase inhibitors, and cytotoxic agents. The term further includes biological anticancer agents and immunotherapeutic drugs (such as immune checkpoint inhibitors) which are usually more specifically targeting the tumor or cancer (targeted therapy). A non-exhaustive overview of anti-cancer agents is included hereafter.

Chemotherapeutic agents

Chemotherapeutic agents may be one of the following compounds, or a derivative or analog thereof: doxorubicin and analogues [such as N-(5,5-diacetoxypent-1-yl)doxorubicin: Farquhar et al. 1998, J Med Chem 41:965-972; epirubicin (4'-epidoxorubicin), 4'-deoxydoxorubicin (esorubicin), 4'-iodo-4'-deoxydoxorubicin, and 4'-O-methyldoxorubicin: Arcamone et al. 1987, Cancer Treatment Rev 14:159-

161 & Giuliani et al. 1980, Cancer Res 40:4682-4687; DOX-F-PYR (pyrrolidine analog of DOX), DOX-F-PIP (piperidine analog of DOX), DOX-F-MOR (morpholine analog of DOX), DOX-F-PAZ (N-methylpiperazine analog of DOX), DOX-F-HEX (hexamehtyleneimine analog of DOX), oxazolinodoxorubicin (3'deamino-3'-N, 4'-O-methylidenodoxorubicin, O-DOX): Denel-Bobrowska et al. 2017, Life Sci 178:1-8)], daunorubicin (or daunomycin) and analogues thereof [such as idarubicin (4'-demethoxydaunorubicin): Arcamone et al. 1987, Cancer Treatment Rev 14:159-161; 4'-epidaunorubicin; analogues with a simplified core structure bound to the monosaccharide daunosamine, acosamine, or 4-amino-2,3,6-trideoxy-L-*threo*-hexopyranose: see compounds 8-13 in Fan et al. 2007, J Organic Chem 72:2917-2928], amrubicin, vinblastine, vincristine, calicheamicin, etoposide, etoposide phosphate, CC-1065 (Boger et al. 1995, Bioorg Med Chem 3:611-621), duocarmycins (such as duocarmycin A and duocarmycin SA; Boger et al. 1995, Proc Natl Acad Sci USA 92:3642-3649), the duocarmycin derivative KW-2189 (Kobayashi et al. 1994, Cancer Res 54:2404-2410), methotrexate, methopterin, aminopterin, dichloromethotrexate, docetaxel, paclitaxel, epithiolone, combretastatin, combretastatin A4 phosphate, dolastatin 10, dolastatin 10 analogues (such as auristatins, e.g. auristatin E, auristatin-PHE, monomethyl auristatin D, monomethyl auristatin E, monomethyl auristatin F; see e.g. Maderna et al. 2014, J Med Chem 57:10527-10534), dolastatin 11, dolastatin 15, topotecan, camptothecin, mitomycin C, porfiromycin, 5-fluorouracil, 6-mercaptopurine, fludarabine, tamoxifen, cytosine arabinoside, adenosine arabinoside, colchicine, halichondrin B, cisplatin, carboplatin, mitomycin C, bleomycin and analogues thereof (e.g. liblomycin, Takahashi et al. 1987, Cancer Treatment Rev 14:169-177), melphalan, chloroquine, cyclosporin A, and maytansine (and maytansinoids and analogues thereof such as analogues comprising a disulfide or thiol substituent: Widdison et al. 2006, J Med Chem 49:4392-4408; maytansin analogs DM1 and DM4). By derivative is intended a compound that results from reacting the named compound with another chemical moiety, and includes a pharmaceutically acceptable salt, acid, base, ester or ether of the named compound.

Other therapeutic agents or drugs include: vindesine, vinorelbine, 10-deacetyltaxol, 7-epi-taxol, baccatin III, 7-xylosyltaxol, isotaxel, ifosfamide, chloroaminophene, procarbazine, chlorambucil, thiophosphoramide, busulfan, dacarbazine (DTIC), geldanamycin, nitroso ureas, estramustine, BCNU, CCNU, fotemustine, streptonigrin, oxaliplatin, methotrexate, aminopterin, raltitrexed, gemcitabine, cladribine, clofarabine, pentostatin, hydroxyureas, irinotecan, topotecan, 9- dimethylaminomethyl-hydroxy-camptothecin hydrochloride, teniposide, amsacrine; mitoxantrone; L-canavanine, THP-adriamycin, idarubicin, rubidazone, pirarubicin, zorubicin, aclarubicin, epiadriamycin (4'epi- adriamycin or epirubicin), mitoxantrone, bleomycins, actinomycins including actinomycin D, streptozotocin, calicheamycin; L- asparaginase; hormones; pure inhibitors of aromatase; androgens, proteasome inhibitors; farnesyl-transferase inhibitors (FTI); epothilones; discodermolide; fostriecin; inhibitors of

tyrosine kinases such as STI 571 (imatinib mesylate); receptor tyrosine kinase inhibitors such as erlotinib, sorafenib, vandetanib, canertinib, PKI 166, gefitinib, sunitinib, lapatinib, EKB-569; Bcr-Abl kinase inhibitors such as dasatinib, nilotinib, imatinib; aurora kinase inhibitors such as VX-680, CYC116, PHA-739358, SU-6668, JNJ-7706621, MLN8054, AZD-1152, PHA-680632; CDK inhibitors such as flavopirodol, seliciclib, E7070, BMS- 387032; MEK inhibitors such as PD184352, U-0126; mTOR inhibitors such as CCI-779 or AP23573; kinesin spindle inhibitors such as ispinesib or MK-0731; RAF/MEK inhibitors such as Sorafenib, CHIR-265, PLX-4032, CI-1040, PD0325901 or ARRY-142886; bryostatin; L-779450; LY333531; endostatins; the HSP 90 binding agent geldanamycin, macrocyclic polyethers such as halichondrin B, eribulin, or an analogue or derivative of any thereof.

Immunotherapy and immunogenic therapy

Immunotherapy in general is defined as a treatment that uses the body's own immune system to help fight a disease, more specifically cancer in the context of the current invention. Immunotherapeutic treatment as used herein refers to the reactivation and/or stimulation and/or reconstitution of the immune response of a mammal towards a condition such as a tumor, cancer or neoplasm evading and/or escaping and/or suppressing normal immune surveillance. The reactivation and/or stimulation and/or reconstitution of the immune response of a mammal in turn in part results in an increase in elimination of tumorous, cancerous or neoplastic cells by the mammal's immune system (anticancer, antitumor or anti-neoplasm immune response; adaptive immune response to the tumor, cancer or neoplasm). If the increase in elimination of tumorous cells is high enough, either expansion of the tumor or cancer will be halted or the tumor or cancer will start to shrink or regress.

Immunotherapeutic agents include antibodies, in particular monoclonal antibodies, employed as (targeted) anti-cancer agents include alemtuzumab (chronic lymphocytic leukemia), bevacizumab (colorectal cancer), cetuximab (colorectal cancer, head and neck cancer), denosumab (solid tumor`s bony metastases), gemtuzumab (acute myelogenous leukemia), ipilumab (melanoma), ofatumumab (chronic lymphocytic leukemia), panitumumab (colorectal cancer), rituximab (Non-Hodgkin lymphoma), tositumomab (Non-Hodgkin lymphoma) and trastuzumab (breast cancer). Other antibodies include for instance abagovomab (ovarian cancer), adecatumumab (prostate and breast cancer), afutuzumab (lymphoma), amatuximab, apolizumab (hematological cancers), blinatumomab, cixutumumab (solid tumors), dacetuzumab (hematologic cancers), elotuzumab (multiple myeloma), farletuzumab (ovarian cancer), intetumumab (solid tumors), muatuzumab (colorectal, lung and stomach cancer), onartuzumab, parsatuzumab, pritumumab (brain cancer), tremelimumab, ublituximab, veltuzumab (non-Hodgkin's lymphoma), votumumab (colorectal tumors), zatuximab and anti-placental growth factor antibodies such as described in WO 2006/099698.

Immunotherapeutic agents of particular interest further include immune checkpoint inhibitors (such as anti-PD-1, anti-PD-L1 or anti-CTLA-4 antibodies; detailed hereinafter), bispecific antibodies bridging a cancer cell and an immune cell, dendritic cell vaccines and so on. Immunotherapy is a promising new area of cancer therapeutics and several immunotherapies are being evaluated preclinically as well as in clinical trials and have demonstrated promising activity (Callahan et al. 2013, J Leukoc Biol 94:41-53; Page et al. 2014, Annu Rev Med 65:185-202). However, not all the patients are sensitive to immune checkpoint blockade and sometimes PD-1 or PD-L1 blocking antibodies accelerate tumor progression. An overview of clinical developments in the field of immune checkpoint therapy is given by Fan et al. 2019 (Oncology Reports 41:3-14). Combinatorial cancer treatments that include chemotherapies can achieve higher rates of disease control by impinging on distinct elements of tumor biology to obtain synergistic antitumor effects. It is now accepted that certain chemotherapies can increase tumor immunity by inducing immunogenic cell death and by promoting escape in cancer immunoediting, such therapies are therefore called immunogenic therapies as they provoke an immunogenic response. Drug moieties known to induce immunogenic cell death include bleomycin, bortezomib, cyclophosphamide, doxorubicin, epirubicin, idarubicin, mafosfamide, mitoxantrone, oxaliplatin, and patupilone (Bezu et al. 2015, Front Immunol 6:187). Other forms of immunotherapy include chimeric antigen receptor (CAR) T-cell therapy in which allogenic T-cells are adapted to recognize a tumoral neo-antigen and oncolytic viruses preferentially infecting and killing cancer cells. Treatment with RNA, e.g. encoding MLKL, is a further means of provoking an immunogenic response (Van Hoecke et al. 2018, Nat Commun 9:3417), as well as vaccination with neo-epitopes (Brennick et al. 2017, Immunotherapy 9:361-371).

Further anti-tumor agents include those described in general terms in the sections “Pharmacological inhibition of a target of interest”, “Pharmacological knock-down of a protein of interest”, and “Genetic inhibition of a target of interest” included hereinafter, and wherein the target or protein of interest can be any known anti-cancer target or protein.

PD1

Aliases of PD1 provided in GeneCards® include PD-1; PDCD1; Programmed Cell Death 1; Systemic Lupus Erythematosus Susceptibility 2; PD-1; CD279; HPD-1; SLEB2; and HPD-L. The genomic locations for the PDCD1 gene are chr2:241,849,881-241,858,908 (in GRCh38/hg38) and chr2:242,792,033-242,801,060 (in GRCh37/hg19). The GenBank reference PD1 mRNA sequence is known under accession no. NM_005018.3. Approved PD1-inhibiting antibodies include nivolumab, pembrolizumab, and cemiplimab; PD1-inhibiting antibodies under development include CT-011 (pidilizumab) and therapy with PD1-inhibiting antibodies is referred to herein as α-PD-1 therapy or α-PD1 therapy. PD1 siRNA and shRNA products are available through e.g. Origene.

PD-L1

Aliases of PD-L1 provided in GeneCards® include CD274, Programmed Cell Death 1 Ligand 1, B7 Homolog 1, B7H1, PDL1, PDCD1 Ligand 1, PDCD1LG1, PDCD1L1, HPD-L1, B7-H1, B7-H, and Programmed Death Ligand 1. The genomic locations for the PDCD1 gene are chr9:5,450,503-5,470,567 (in GRCh38/hg38) and chr9:5,450,503-5,470,567 (in GRCh37/hg19). The GenBank reference PD1 mRNA sequence is known under accession no. NM_001267706.1, NM_001314029.2 and NM_014143.4. Approved PD-L1-inhibiting antibodies include atezolizumab, avelumab, and durvalumab. PD-L1 siRNA and shRNA products are available through e.g. Origene.

CTLA4

Aliases of CTLA4 provided in GeneCards® include Cytotoxic T-Lymphocyte Associated Protein 4; CTLA-4; CD152; Insulin-Dependent Diabetes Mellitus 12; Cytotoxic T-Lymphocyte Protein 4; Celiac Disease 3; GSE; Ligand And Transmembrane Spliced Cytotoxic T Lymphocyte Associated Antigen 4; Cytotoxic T Lymphocyte Associated Antigen 4 Short Spliced Form; Cytotoxic T-Lymphocyte-Associated Serine Esterase-4; Cytotoxic T-Lymphocyte-Associated Antigen 4; CELIAC3; IDDM12; ALPS5; and GRD4. The genomic locations for the CTLA4 gene are chr2:203,867,771-203,873,965 (in GRCh38/hg38) and chr2:204,732,509-204,738,683 (in GRCh37/hg19). The GenBank reference CTLA4 mRNA sequences are known under accession nos. NM_001037631.3 and NM_005214.5. Approved CTLA4-inhibiting antibodies include ipilumab; CTLA4-inhibiting antibodies under development include tremelimumab. CTLA4 siRNA and shRNA products are available through e.g. Origene.

In any of the above methods of the invention, the class A plexin biomarker can in particular be human plexin A1, human plexin A2, human plexin A3 or human plexin A4, or a combination thereof. In particular, the class A plexin biomarker can be human plexin A1, human plexin A2 or human plexin A4 or a combination thereof. In particular, the class A plexin biomarker can be human plexin A2 or human plexin A4 or a combination thereof. More in particular, the class A plexin biomarker is in one embodiment human plexin A4.

In any of the above methods of the invention, the fluid sample can in particular be selected from blood, urine, saliva, or a fraction of any thereof. Fractions of blood include serum and plasma. A fraction of any sample can further refer to e.g. a cellular fractions (e.g. T-cell fraction), a nucleic acid fraction, or a nucleic acid fraction isolated from a cellular fraction. In one embodiment to the above methods, the fluid sample is a blood sample. In one embodiment to the above methods, the sample is a fraction of a blood sample, more in particular a cellular fraction of a blood sample, more in particular the CD8+ T-cell or cytotoxic T cell (CTL) fraction of a blood sample. In one embodiment, the fraction of a blood sample is a nucleic acid

fraction isolated from the CD8+ T-cell or CTL fraction of a blood sample, more in particular a mRNA nucleic acid fraction isolated from the CD8+ T-cell or CTL fraction of a blood sample. In one embodiment, the fraction of a blood sample is a protein sample or protein fraction from the CD8+ T-cell or CTL fraction of a blood sample.

CD8+ T cells or CTLs are a type of T lymphocytes also known as cytotoxic T lymphocytes, T-killer cells, cytolytic T cells, or killer T cells.

Thus, any of the above methods of the invention can comprise a further step of enriching T-cells from the fluid sample(s) such as to obtain an enriched sample or to obtain the T-cell fraction from the fluid sample(s). Quantifying, determining, assessing, or measuring the amount of the biomarker can then be performed for said enriched sample or for the T-cell fraction obtained from the fluid sample(s). In particular, quantifying, determining, assessing, or measuring the amount of the nucleic acids of, corresponding to, or encoding the biomarker can then be performed for said enriched sample, or for the T-cell fraction obtained from the fluid sample(s), or for the nucleic acids isolated or obtained from said enriched sample or T-cell fraction. In one embodiment, the T-cell fraction is the CD8+ T-cell or CTL fraction.

In any of the above methods of the invention, quantifying, determining, assessing, or measuring the amount of the biomarker can in particular be quantifying, determining, assessing, or measuring the amount of a nucleic acid of said biomarker or corresponding to or encoding said biomarker. Said nucleic acid can be DNA (such as comprised in cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA)) or RNA, such as mRNA.

In any of the above methods of the invention, quantifying, determining, assessing, or measuring the amount of the biomarker can in particular be quantifying, determining, assessing, or measuring the amount of biomarker protein.

In any of the above methods of the invention, the tumor can be a solid tumor or an inflamed tumor. In particular the tumor can be melanoma. Further in particular, the melanoma tumor is at a resectable stage disease level (e.g. up to stage III resectable disease). Further in particular, the melanoma is not at non-resectable disease level (e.g. stage III or stage IV non-resectable disease).

In any of the above methods of the invention, the first sample refers to a sample taken not before but at the time of administration of the anti-tumor therapy. It is easily understood that “before” and “at the time” in this context are the same, in both instances, the effect of the (1st or other dose of) anti-tumor

therapy has not started and has not yet (further) changed or affected the tumor or immune system of the subject having the tumor and receiving the anti-tumor therapy. In the same context, the second sample is obtained after a given dose of anti-tumor therapy, meaning that a minimum of time is interspersing the timing of administration of said given dose and the timing of having obtained the second sample, i.e., sufficient time in order for the said given dose to have started its effect (at least in responders).

In any of the above methods of the invention, the anti-tumor therapy can be a therapy comprising or consisting of anti-tumor immunotherapy. In particular, the anti-tumor immunotherapy can be immune checkpoint inhibitor therapy. More in particular, the immune checkpoint inhibitor therapy can comprise administration of an inhibitor of PD-1, an inhibitor of PD-L1, and/or an inhibitor of CTLA4. More in particular, the immune checkpoint inhibitor therapy is a therapy with an inhibitor of PD-1, with an inhibitor of CTLA4, or with a combination of an inhibitor of PD-1 and an inhibitor of CTLA4.

In any of the above methods of the invention, the anti-tumor therapy is in one embodiment a neo-adjuvant anti-tumor therapy (therapy provided to a subject having cancer prior to the main treatment such as surgery/surgical resection of the tumor or cancer or such a radiation therapy). Neo-adjuvant anti-tumor therapy is usually provide in order to reduce the size of a tumor and/or to kill tumor cells that have spread. Thus, in any of the above methods, response to neoadjuvant anti-tumor therapy is monitored, determined, assessed, measured, or predicted.

Thus, in a further alternative aspect, the invention relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such methods comprising one or more of the following steps:

- obtaining a first blood sample and a second blood sample from said subject, wherein said first sample is obtained at or before at least one administration of an anti-tumor therapy, and said second sample is obtained after at least one administration of an anti-tumor therapy comprising immune checkpoint inhibitory therapy;
- enriching T-cells from said first and second sample to obtain an enriched first and second sample; and
- quantifying the amount of human plxnA2 RNA and/or of human plxnA4 RNA in said enriched first and second sample;

wherein a reduction of the amount of human plxnA2 RNA and/or human plxnA4 RNA in said second sample compared to said first sample indicates that the subject is responsive to said anti-tumor therapy

or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of human plxnA2 RNA and/or human plxnA4 RNA in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

In an alternative aspect, the invention relates to methods for monitoring the response of a subject having a tumor to an anti-tumor therapy comprising immune checkpoint inhibitory therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy comprising immune checkpoint inhibitory therapy, such methods comprising quantifying the amount of plxnA2 RNA and/or plxnA4 RNA in T-cells enriched from a first and a second fluid sample such as a blood sample;

wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy and said first sample is obtained from the subject at or before administration of the at least one dose of the anti-tumor therapy or said first sample is a control or reference sample; and

wherein a reduction of the amount of human plxnA2 RNA and/or human plxnA4 RNA in said second sample compared to said first sample indicates that the subject is responsive to said anti-tumor therapy or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of human plxnA2 RNA and/or human plxnA4 RNA in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Such methods can obviously be adapted as described hereinabove such that the amount of human plxnA2 RNA and/or human plxnA4 RNA can be compared with suitable control or reference samples or values assembled for responders and non-responders to the anti-tumor therapy. In such adapted methods, not a reduction of the amount of human plxnA2 RNA and/or human plxnA4 RNA is decisive for deciding whether or not the subject or the subject's tumor or cancer is responsive to the anti-tumor therapy.

In yet a further aspect, the invention relates to methods for monitoring response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such methods comprising one or more of the following steps:

- obtaining a first blood sample and a second blood sample from said subject, wherein said first sample is obtained at or before at least one administration of an anti-tumor therapy, and said second sample is obtained after at least one administration of an anti-tumor therapy comprising immune checkpoint inhibitory therapy;
- enriching CD8+T-cells or CTLs from said first and second sample to obtain an enriched first and second sample; and
- quantifying the amount of human plxnA4 RNA in said enriched first and second sample, or in said CD8+ T-cells or CTLs enriched from said first and second sample;

wherein a reduction of the amount of human plxnA4 RNA in said second sample compared to said first sample indicates that the subject is responsive to said anti-tumor therapy or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of human plxnA4 RNA in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

In one embodiment, the anti-tumor therapy is immune checkpoint inhibitor therapy.

In an alternative aspect, the invention relates to methods for monitoring the response of a subject having a tumor to an anti-tumor therapy comprising immune checkpoint inhibitory therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy comprising immune checkpoint inhibitory therapy, such methods comprising quantifying the amount of plxnA4 RNA in CD8+T-cells or CTLs enriched from a first and a second fluid sample such as a blood sample (or in said CD8+ T-cells or CTLs enriched from said first and second sample);

wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy and said first sample is obtained from the subject at or before administration of the at least one dose of the anti-tumor therapy or said first sample is a control or reference sample; and

wherein a reduction of the amount of human plxnA4 RNA in said second sample compared to said first sample indicates that the subject is responsive to said anti-tumor therapy or is indicative or predictive for the subject to be or being responsive to said anti-tumor therapy; and/or wherein the absence of a reduction of the amount of human plxnA4 RNA in the second sample compared to the first sample indicates that the subject is not responsive to the anti-tumor therapy or is indicative or predictive for the subject not to be or not being responsive to said anti-tumor therapy.

Such methods can obviously be adapted as described hereinabove such that the amount of human plxnA4 RNA can be compared with suitable control or reference samples or values assembled for

responders and non-responders to the anti-tumor therapy. In such adapted methods, not a reduction of the amount of human plxnA4 RNA is decisive for deciding whether or not the subject or the subject's tumor or cancer is responsive to the anti-tumor therapy.

In one embodiment, the anti-tumor therapy is immune checkpoint inhibitor therapy.

As explained in Example 1 herein, changes in plexin A biomarker levels are detectable early after the administration of the first dose of the anti-tumor therapy. Whereas the clinical outcome of a subject having a tumor being a responder or non-responder to the anti-tumor therapy at least takes 3 months or more, the changes in plexin A biomarker levels in circulating T-cells are already predictive for response or non-response 3 weeks or less following the administration of a dose of the anti-tumor therapy. The plexin A biomarker of the current invention thus provides a way for early detection of response to an anti-tumor therapy in a non-invasive manner. Therefore, in any of the above-described methods the said second fluid sample can be obtained from the subject two to four weeks (such as 2 weeks, 3 weeks or 4 weeks, or 14 to 30 days) after administration of a dose of the anti-tumor therapy. Herein, the administration of a dose of anti-tumor therapy can be the administration of a first dose of anti-tumor therapy. As it is known that not all patients respond evenly quick to immune checkpoint inhibitor therapy, repetitive determination of plexin A biomarker levels at different time points is not excluded, thus the administration of a dose of anti-tumor therapy can likewise be the administration of a second, third, etc. dose of anti-tumor therapy.

Any of the above described methods can further comprise the step of continuing the anti-tumor therapy in a subject determined to be responsive to the anti-tumor therapy, or continuing administering the anti-tumor therapy to a subject determined to be responsive to the anti-tumor therapy. Alternatively, any of the above described methods can further comprise the step of discontinuing the anti-tumor therapy in a subject determined to be non-responsive to the anti-tumor therapy, or discontinuing administering the anti-tumor therapy to a subject determined to be non-responsive to the anti-tumor therapy. In one embodiment, the anti-tumor therapy is a therapy excluding or not comprising a plexin class A antagonist or inhibitor. In one embodiment, the anti-tumor therapy is a therapy comprising an immune checkpoint inhibitor. In one embodiment, the anti-tumor therapy is a therapy comprising an immune checkpoint inhibitor and excluding or not comprising a plexin class A antagonist or inhibitor.

The invention further relates to a plexin class A antagonist or inhibitor for use in (a method of) treating a subject having a tumor, or for use in treating, inhibiting or inhibiting progression of a tumor in a subject, wherein the subject has been indicated to be not responsive to a different anti-tumor therapy using any

of the above methods. The different anti-tumor therapy in particular is any anti-tumor therapy not including, not comprising, or excluding administration of a plexin class A antagonist or inhibitor.

Alternatively, the invention relates to a plexin class A antagonist or inhibitor for use in (a method of) treating a subject having a tumor, or for use in treating, inhibiting or inhibiting progression of a tumor in a subject, comprising performing any of the above methods; and administering the plexin class A antagonist or inhibitor to the subject when the amount of plexin class A biomarker indicates or predicts that the subject is not responsive to a first anti-tumor therapy or is indicative or predictive for the subject to be or not being non-responsive to a first anti-tumor therapy.

Alternatively, the invention relates to a plexin class A antagonist or inhibitor for use in (a method of) treating a subject having a tumor, or for use in treating, inhibiting or inhibiting progression of a tumor in a subject, comprising monitoring the response of the subject to a first anti-tumor therapy according to any of the above methods; and administering the plexin class A antagonist or inhibitor to the subject when the amount of plexin class A biomarker indicates that the subject is not responsive to said first anti-tumor therapy or is indicative or predictive for the subject to be non-responsive or not being responsive to said first anti-tumor therapy.

Alternatively, the invention relates to a plexin class A antagonist or inhibitor for use in (a method of) treating a subject having a tumor, or for use in treating, inhibiting or inhibiting progression of a tumor in a subject, comprising monitoring response of the subject to a first anti-tumor therapy according to any of the above methods; and replacing or combining said first anti-tumor therapy with a second anti-tumor therapy when the amount of plexin class A biomarker indicates that the subject is not responsive to said first anti-tumor therapy or is indicative or predictive for the subject to be non-responsive to or not being responsive to said first anti-tumor therapy. In particular, said second anti-tumor therapy comprises administration of a plexin class A antagonist or inhibitor.

Alternatively, the invention relates to methods for treating a subject having a tumor, the method comprising monitoring response of the subject to a first anti-tumor therapy according to the any of the above methods, and if any of such method for monitoring response indicates that said subject is not responsive to said first anti-tumor therapy, then replacing or combining said first anti-tumor therapy with a second anti-tumor therapy. In particular, said second anti-tumor therapy comprises administration of a plexin class A antagonist or inhibitor.

In the above, the first anti-tumor therapy is in particular a therapy not including, not comprising, or excluding administration of a plexin class A antagonist or inhibitor, such as a plexin A4 antagonist or inhibitor. In one embodiment, the first anti-tumor therapy is a therapy comprising an immune checkpoint inhibitor.

In the above, the administration of a plexin class A antagonist or inhibitor to said subject, in particular of a therapeutically effective dose of the plexin class A antagonist or inhibitor, is treating the subject having the tumor, or in treating, inhibiting or inhibiting progression of a tumor in the subject.

Tumor, cancer, neoplasm

The terms tumor and cancer are sometimes used interchangeably but can be distinguished from each other. A tumor refers to "a mass" which can be benign (more or less harmless) or malignant (cancerous). A cancer is a threatening type of tumor. A tumor is sometimes referred to as a neoplasm: an abnormal cell growth, usually faster compared to growth of normal cells. Benign tumors or neoplasms are non-malignant/non-cancerous, are usually localized and usually do not spread/metastasize to other locations. Because of their size, they can affect neighboring organs and may therefore need removal and/or treatment. A cancer, malignant tumor or malignant neoplasm is cancerous in nature, can metastasize, and sometimes re-occurs at the site from which it was removed (relapse). The initial site where a cancer starts to develop gives rise to the primary cancer. When cancer cells break away from the primary cancer ("seed"), they can move (via blood or lymph fluid) to another site even remote from the initial site. If the other site allows settlement and growth of these moving cancer cells, a new cancer, called secondary cancer, can emerge ("soil"). The process leading to secondary cancer is also termed metastasis, and secondary cancers are also termed metastases. For instance, liver cancer can arise as primary cancer, but can also be a secondary cancer originating from a primary breast cancer, bowel cancer or lung cancer; some types of cancer show an organ-specific pattern of metastasis. Most cancer deaths are in fact caused by metastases, rather than by primary tumors (Chambers et al. 2002, Nature Rev Cancer2:563-572).

Sample / biological sample / fluid sample

A sample can be any biological sample isolated from or obtained from a subject. For example, a sample can comprise, without limitation, bodily fluid, whole blood, serum, plasma, synovial fluid, lymphatic fluid, ascites fluid, interstitial or extracellular fluid, the fluid in spaces between cells, including gingival crevicular fluid, cerebrospinal fluid, saliva, mucous, sputum, phlegm, smegma, seminal fluid, ejaculate, sweat, tears, urine, fluid from nasal brushings, colonic washing fluid, fluid from a pap smear, vaginal fluid, vaginal flushing fluid, fluid from a hydrocele, pleural fluid, bronchoalveolar lavage fluid, discharge fluid from the nipple, aspiration fluid from a part of the body, colostrum, breast milk, ventricular fluid, any other bodily fluids. A bodily fluid can include saliva, blood, or serum. A sample can comprise a volume of plasma containing cell free DNA molecules. A sample may comprise a volume of plasma sufficient to achieve a given read depth. A volume of sampled plasma may be at least 0.5 milliliters (mL), 1 mL, 5 mL

10 mL, 20 mL, 30 mL, or 40 mL. A volume of sampled plasma at most 0.5 mL, 1 mL, 5 mL 10 mL, 20 mL, 30 mL, or 40 mL. A volume of sampled plasma may be 5 to 20 mL. A volume of sampled plasma may be 10 ml to 20 mL

Isolation or extraction of polynucleotides may be performed through collection of bodily fluids using a variety of techniques. In some cases, collection may comprise aspiration of a bodily fluid from a subject using a syringe. In other cases collection may comprise pipetting or direct collection of fluid into a collecting vessel. In yet further cases, it may comprise collecting fluid with a small brush or brush-like device, and washing the brush or brush-like device in a suitable buffer to release polynucleotides contained in the collected fluid. A sample may be comprising cells. For the isolation of cfDNA, cells are removed from the sample e.g. by precipitation, centrifugation or filtration.

After collection of bodily fluid, and, when required, after removal of cells, polynucleotides such as cfDNA may be isolated or extracted using a variety of techniques utilized in the art. In some cases, cell-free DNA may be isolated, extracted and prepared using commercially available kits such as the Qiagen Qiamp® Circulating Nucleic Acid Kit protocol. In other examples, Qiagen Qubit™ dsDNA HS Assay kit protocol, Agilent™ DNA 1000 kit, or TruSeq™ Sequencing Library Preparation; Low-Throughput (LT) protocol may be used. Further DNA extraction products include the DNeasy Blood and Tissue extraction kit (Qiagen) and automated systems for DNA extraction such as the QiaSymphony (Qiagen) and Chemagen 360 (PerkinElmer).

In some instances, a plasma sample is treated with proteinase K (to degrade proteins/proteinaceous compounds present in the sample) and DNA is precipitated with isopropanol and subsequently captured on e.g. a Qiagen column. The DNA then can be eluted by an eluent such as water or Tris-EDTA (TE) elution buffer. In some embodiments, a portion of the DNA can be selected based on size (e.g., DNA of 500 nucleotides or fewer in length), for example, using Solid Phase Reversible Immobilization (SPRI) beads, such as AgenCourt®AMPure® beads. In some embodiments, the DNA can be resuspended in a smaller volume. Approximately 5 ng of DNA may be equivalent to about 1500 haploid genome equivalents ("HGE").

Gene expression level

The term "level of expression" or "expression level" generally refers to the amount of an expressed biomarker in a biological sample. "Expression" generally refers to the process by which information (e.g., gene- encoded and/or epigenetic information) is converted into the structures present and operating in the cell. Therefore, as used herein, "expression" may refer to transcription into a polynucleotide, translation into a polypeptide, or even polynucleotide and/or polypeptide modifications (e.g., posttranslational modification of a polypeptide). Fragments of the transcribed polynucleotide, the

translated polypeptide, or polynucleotide and/or polypeptide modifications (e.g., posttranslational modification of a polypeptide) are also regarded as expressed whether they originate from a transcript generated by alternative splicing or a degraded transcript, or from a post-translational processing of the polypeptide, e.g., by proteolysis. "Expressed genes" include those that are transcribed into a polynucleotide as mRNA and then translated into a polypeptide, and also those that are transcribed into RNA but not translated into a polypeptide (for example, transfer and ribosomal RNAs, long non-coding RNA, microRNA or miRNA).

"Increased expression," "increased expression level," "increased levels," "elevated expression," "elevated expression levels," or "elevated levels" refers to an increased expression or increased levels of a biomarker in an individual relative to a control, such as an individual or individuals who do not have the disease or disorder (e.g., cancer), an internal control (e.g., a housekeeping biomarker), a median expression level of the biomarker in samples from a group/population of patients, or relative to an expression level of the biomarker in samples taken before onset of a certain therapy.

The term "detection" includes any means of detecting, including direct and indirect detection. The term "biomarker" as used herein refers to an indicator molecule or set of molecules (e.g., predictive, diagnostic, and/or prognostic indicator), which can be detected in a sample. The biomarker may be a predictive biomarker and serve as an indicator of the likelihood of sensitivity or benefit of a patient having a particular disease or disorder (e.g., a proliferative cell disorder (e.g., cancer)) to treatment. Biomarkers include, but are not limited to, polynucleotides (e.g., DNA and/or RNA (e.g., mRNA)), polynucleotide copy number alterations (e.g., DNA copy numbers), polypeptides, polypeptide and polynucleotide modifications (e.g., post-translational modifications, nucleotide substitutions, nucleotide insertions or deletions (indels)), carbohydrates, and/or glycolipid-based molecular markers. In some embodiments, a biomarker is a gene. The "amount" or "level" of a biomarker, as used herein, is a detectable level in a biological sample. These can be measured by methods known to one skilled in the art and also disclosed herein.

Any gene detection or gene expression detection method is starting from an analyte nucleic acid (i.e. the nucleic acid of interest (which does not necessarily need to be the whole nucleic acid of interest, parts of such nucleic acids can suffice for determining expression) and of which the amount is to be determined) and may be defined as comprising one or more of, for instance,

- a step of isolating RNA from a biological sample (wherein a fraction of the isolated RNA is the analyte strand); the biological sample obtained from the test subject;
- a step of reverse transcribing the RNA obtained from the biological sample into DNA;
- a step of amplifying the isolated DNA; and/or

- a step of quantifying the isolated RNA, the DNA obtained after reverse transcription, or the amplified DNA.

In case an amplified DNA is quantified, this quantification step can be performed concurrent with the amplification of the DNA, or is performed after the amplification of the DNA.

The quantification of gene expression or the determination of gene expression levels may be based on at least one of an amplification reaction, a sequencing reaction, a melting reaction, a hybridization reaction or a reverse hybridization reaction.

Detection and quantification of gene expression

The invention covers methods for detecting the presence of nucleic acids corresponding to one or more class A plexins as defined herein in a biological sample and/or methods for determining or detecting the expression level of one or more class A plexins as defined herein, wherein said methods comprise the step of detecting the presence of a class A plexin of interest nucleic acid or expression level of a class A plexin of interest. In any of these methods the detection can comprise a step such as a nucleic acid amplification reaction, a nucleic acid sequencing reaction, a melting reaction, a hybridization reaction to a nucleic acid, or a reverse hybridization reaction to a nucleic acid, or a combination of such steps.

Often one or more artificial, man-made, non-naturally occurring oligonucleotide is used in such method. In particular, such oligonucleotides can comprise besides ribonucleic acid monomers or deoxyribonucleic acid monomers: one or more modified nucleotide bases, one or more modified nucleotide sugars, one or more labelled nucleotides, one or more peptide nucleic acid monomers, one or more locked nucleic acid monomers, the backbone of such oligonucleotide can be modified, and/or non-glycosidic bonds may link two adjacent nucleotides. Such oligonucleotides may further comprise a modification for attachment to a solid support, e.g., an amine-, thiol-, 3-'propanolamine or acrydite-modification of the oligonucleotide, or may comprise the addition of a homopolymeric tail (for instance an oligo(dT)-tail added enzymatically via a terminal transferase enzyme or added synthetically) to the oligonucleotide. If said homopolymeric tail is positioned at the 3'-terminus of the oligonucleotide or if any other 3'-terminal modification preventing enzymatic extension is incorporated in the oligonucleotide, the priming capacity of the oligonucleotide can be decreased or abolished. Such oligonucleotides may also comprise a hairpin structure at either end. Terminal extension of such oligonucleotide may be useful for, e.g., specifically hybridizing with another nucleic acid molecule (e.g. when functioning as capture probe), and/or for facilitating attachment of said oligonucleotide to a solid support, and/or for modification of said tailed oligonucleotide by an enzyme, ribozyme or DNAzyme. Such oligonucleotides may be modified in order to detect (the levels of) a target nucleotide sequence and/or to facilitate in any way such detection. Such modifications include labelling with a single label, with two different labels (for instance two

fluorophores or one fluorophore and one quencher), the attachment of a different 'universal' tail to two probes or primers hybridizing adjacent or in close proximity to each other with the target nucleotide sequence, the incorporation of a target-specific sequence in a hairpin oligonucleotide (for instance Molecular Beacon-type primer), the tailing of such a hairpin oligonucleotide with a 'universal' tail (for instance Sunrise-type probe and Amplifluor TM -type primer). A special type of hairpin oligonucleotide incorporates in the hairpin a sequence capable of hybridizing to part of the newly amplified target DNA. Amplification of the hairpin is prevented by the incorporation of a blocking nonamplifiable monomer (such as hexethylene glycol). A fluorescent signal is generated after opening of the hairpin due to hybridization of the hairpin loop with the amplified target DNA. This type of hairpin oligonucleotide is known as scorpion primers (Whitcombe et al. 1999, Nat Biotechnol 17:804-807). Another special type of oligonucleotide is a padlock oligonucleotide (or circularizable, open circle, or C-oligonucleotide) that are used in RCA (rolling circle amplification). Such oligonucleotides may also comprise a 3'-terminal mismatching nucleotide and/or, optionally, a 3'-proximal mismatching nucleotide, which can be particularly useful for performing polymorphism-specific PCR and LCR (ligase chain reaction) or any modification of PCR or LCR. Such oligonucleotide may can comprise or consist of at least and/or comprise or consist of up to 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200 or more contiguous nucleotides.

The analyte nucleic acid, in particular the analyte nucleic acid of a class A plexin of interest can be any type of nucleic acid, which will be dependent on the manipulation steps (such as isolation and/or purification and/or duplication, multiplication or amplification) applied to the nucleic acid of the gene of interest in the biological sample; as such it can be DNA, RNA, cDNA, may comprise modified nucleotides, or may be hybrids of DNA and/or RNA and/or modified nucleotides, and can be single- or double-stranded or may be a triplex-forming nucleic acid.

The artificial, man-made, non-naturally occurring oligonucleotide(s) as applied in the above detection methods can be probe(s) or a primer(s), or a combination of both.

A probe capable of specifically hybridizing with a target nucleic acid is an oligonucleotide mainly hybridizing to one specific nucleic acid sequence in a mixture of many different nucleic acid sequences. Specific hybridization is meant to result, upon detection of the specifically formed hybrids, in a signal-to-noise ratio (wherein the signal represents specific hybridization and the noise represents unspecific hybridization) sufficiently high to enable unambiguous detection of said specific hybrids. In a specific case specific hybridization allows discrimination of up to a single nucleotide mismatch between the probe and the target nucleic acids. Conditions allowing specific hybridization generally are stringent but can obviously be varied depending on the complexity (size, GC-content, overall identity, etc.) of the

probe(s) and/or target nucleic acid molecules. Specificity of a probe in hybridizing with a nucleic acid can be improved by introducing modified nucleotides in said probe.

A primer capable of directing specific amplification of a target nucleic acid is the at least one oligonucleotide in a nucleic acid amplification reaction mixture that is required to obtain specific amplification of a target nucleic acid. Nucleic acid amplification can be linear or exponential and can result in an amplified single nucleic acid of a single- or double-stranded nucleic acid or can result in both strands of a double-stranded nucleic acid. Specificity of a primer in directing amplification of a nucleic acid can be improved by introducing modified nucleotides in said primer. The fact that a primer does not have to match exactly with the corresponding template or target sequence to warrant specific amplification of said template or target sequence is amply documented in literature (for instance: Kwok et al. 1990, Nucl Acids Res 18:999-1005. Primers as short as 8 nucleotides in length have been applied successfully in directing specific amplification of a target nucleic acid molecule (e.g. Majzoub et al. 1983, J Biol Chem 258:14061-14064).

A nucleotide is meant to include any naturally occurring nucleotide as well as any modified nucleotide wherein said modification can occur in the structure of the nucleotide base (modification relative to A, T, G, C, or U) and/or in the structure of the nucleotide sugar (modification relative to ribose or deoxyribose). Any of the modifications can be introduced in a nucleic acid or oligonucleotide to increase/decrease stability and/or reactivity of the nucleic acid or oligonucleotide and/or for other purposes such as labelling of the nucleic acid or oligonucleotide. Modified nucleotides include phophorothioates, alkylphophorothioates, methylphosphonate, phosphoramidate, peptide nucleic acid monomers and locked nucleic acid monomers, cyclic nucleotides, and labelled nucleotides (i.e. nucleotides conjugated to a label which can be isotopic (<32>P, <35>S, etc.) or non-isotopic (biotin, digoxigenin, phosphorescent labels, fluorescent labels, fluorescence quenching moiety, etc.)). Other modifications are described higher (see description on oligonucleotides).

Nucleotide acid amplification is meant to include all methods resulting in multiplication of the number of a target nucleic acid. Nucleotide sequence amplification methods include the polymerase chain reaction (PCR; DNA amplification), strand displacement amplification (SDA; DNA amplification), transcription-based amplification system (TAS; RNA amplification), self-sustained sequence replication (3SR; RNA amplification), nucleic acid sequence-based amplification (NASBA; RNA amplification), transcription-mediated amplification (TMA; RNA amplification), Qbeta-replicase-mediated amplification and run-off transcription. During amplification, the amplified products can be conveniently labeled either using labeled primers or by incorporating labeled nucleotides.

The most widely spread nucleotide sequence amplification technique is PCR. The target DNA is exponentially amplified. Many methods rely on PCR including AFLP (amplified fragment length

polymorphism), IRS-PCR (interspersed repetitive sequence PCR), iPCR (inverse PCR), RAPD (rapid amplification of polymorphic DNA), RT-PCR (reverse transcription PCR) and real-time PCR. RT-PCR can be performed with a single thermostable enzyme having both reverse transcriptase and DNA polymerase activity (Myers et al. 1991, Biochem 30:7661-7666). Alternatively, a single tube-reaction with two enzymes (reverse transcriptase and thermostable DNA polymerase) is possible (Cusi et al. 1994, Biotechniques 17:1034-1036).

Solid phases, solid matrices or solid supports on which molecules, e.g., nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, may be bound (or captured, absorbed, adsorbed, linked, coated, immobilized; covalently or non-covalently) comprise beads or the wells or cups of microtiter plates, or may be in other forms, such as solid or hollow rods or pipettes, particles, e.g., from 0.1 µm to 5 mm in diameter (e.g. "latex" particles, protein particles, or any other synthetic or natural particulate material), microspheres or beads (e.g. protein A beads, magnetic beads). A solid phase may be of a plastic or polymeric material such as nitrocellulose, polyvinyl chloride, polystyrene, polyamide, polyvinylidene fluoride or other synthetic polymers. Other solid phases include membranes, sheets, strips, films and coatings of any porous, fibrous or bibulous material such as nylon, polyvinyl chloride or another synthetic polymer, a natural polymer (or a derivative thereof) such as cellulose (or a derivative thereof such as cellulose acetate or nitrocellulose). Fibers or slides of glass, fused silica or quartz are other examples of solid supports. Paper, e.g., diazotized paper may also be applied as solid phase. Clearly, molecules such as nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, may be bound, captured, absorbed, adsorbed, linked or coated to any solid phase suitable for use in hybridization assay (irrespective of the format, for instance capture assay, reverse hybridization assay, or dynamic allele-specific hybridization (DASH)). Said molecules, such as nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, can be present on a solid phase in defined zones such as spots or lines. Such solid phases may be incorporated in a component such as a cartridge of e.g. an assay device. Any of the solid phases described above can be developed, e.g. automatically developed in an assay device.

Alternatively, analyte nucleic acids are divided or dispersed in droplets

Quantification of amplified DNA can be performed concurrent with or during the amplification. Techniques include real-time PCR or (semi-)quantitative polymerase chain reaction (qPCR). One common method includes measurement of a non-sequence specific fluorescent dye (e.g. SYBR Green) intercalating in any double-stranded DNA. Quantification of multiple amplicons with different melting points can be followed simultaneously by means of following or analyzing the melting reaction (melting curve analysis or melt curve analysis; which can be performed at high resolution, see, e.g. Wittwer et al.

2003, Clin Chem 843-860; an alternative method is denaturing gel gradient electrophoresis, DGGE; both methods were compared in e.g. Tindall et al. 2009, Hum Mutat 30:857-859).

Another common method includes measurement of sequence-specific labelled probe bound to its complementary sequence; such probe also carries a quencher and the label is only measurable upon exonucleolytic release from the probe (hydrolysis probes such as TaqMan probes) or upon hybridization with the target sequence (hairpin probes such as molecular beacons which carry an internally quenched fluorophore whose fluorescence is restored upon unfolding the hairpin). This latter method allows for multiplexing by e.g. using mixtures of probes each tagged with a different label e.g. fluorescing at a different wavelength.

Exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probes also allow for multiplexing. The hybridization-sensitive fluorescence emission of ECHO probes and the further modification of probes have made possible multicolor RNA imaging in living cells and facile detection of gene polymorphisms (Okamoto 2011, Chem Soc Rev, 40:5815–5828).

Other methods of quantifying expression include SAGE (Serial Analysis of Gene Expression) and MPSS (Massively Parallel Signature Sequencing), each involving reverse-transcription of RNA.

With "assaying" or "determining" or “detecting” and the like (e.g. assessing, measuring) is meant that a biological sample, suspected of comprising a target nucleic acid (such as a nucleic acid of interest as described herein), is processed as to generate a readable signal in case the target nucleic acid is actually present in the biological sample. Such processing may include, as described above, a step of producing an analyte nucleic acid. Simple detection of a produced readable signal indicates the presence of a target or analyte nucleic acid in the biological sample. When in addition the amplitude of the produced readable signal is determined, this allows for quantification of levels of a target or analyte nucleic acid as present in a biological sample.

In particular, the readable signal may be a signal-to-noise ratio (wherein the signal represents specific detection and the noise represents unspecific detection) of an assay optimized to yield signal-to-noise ratios sufficiently high to enable unambiguous detection and/or quantification of the target nucleic acid. The noise signal, or background signal, can be determined e.g. on biological samples not comprising the target or analyte nucleic acid of interest, e.g. control samples, or comprising the required reference level of the target or analyte nucleic acid of interest, e.g. reference samples. Such noise or background signal may also serve as comparator value for determining an increase or decrease of the level of a target or analyte nucleic acid in the biological sample, e.g. in a biological sample taken from a subject suffering from a disease or disorder, further e.g. before start of a treatment and during treatment.

The readable signal may be produced with all required components in solution or may be produced with some of the required components in solution and some bound to a solid support. Said signals include,

e.g., fluorescent signals, (chemi)luminescent signals, phosphorescence signals, radiation signals, light or color signals, optical density signals, hybridization signals, mass spectrometric signals, spectrometric signals, chromatographic signals, electric signals, electronic signals, electrophoretic signals, real-time PCR signals, PCR signals, LCR signals, Invader-assay signals, sequencing signals (by any method such as Sanger dideoxy sequencing, pyrosequencing, 454 sequencing, single-base extension sequencing, sequencing by ligation, sequencing by synthesis, "next-generation" sequencing (NGS)(van Dijk et al. 2014, Trends Genet 30:418-426)), melting curve signals, single-pore sequencing etc. An assay may be run automatically or semi-automatically in an assay device. In view of its relatively low costs compared to e.g. very costly cancer therapies, NGS is finding its way to routine clinical care (Ratner 2018, Nature Biotechnol 36:484).

Specific hybridization of an oligonucleotide (whether or not comprising one or more modified nucleotides) to its target sequence is to be understood to occur under stringent conditions as generally known in the art (e.g. Sambrook et al. 1989. Molecular Cloning. A laboratory manual. CSHL Press). However, depending to the hybridization solution (SSC, SSPE, etc.), oligonucleotides should be hybridized at their appropriate temperature in order to attain sufficient specificity. In order to allow hybridization to occur, the target nucleic acid molecules are generally thermally, chemically (e.g. by NaOH) or electrochemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridization is influenced by conditions such as temperature, salt concentration and hybridization buffer composition. High stringency conditions for hybridization include high temperature and/or low salt concentration (salts include NaCl and Na3-citrate) and/or the inclusion of formamide in the hybridization buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridization buffer and/or exclusion of compounds such as dextran sulfate or polyethylene glycol (promoting molecular crowding) from the hybridization buffer. Conventional hybridization conditions are described in e.g. Sambrook et al. 1989 (Molecular Cloning. A laboratory manual. CSHL Press) but the skilled craftsman will appreciate that numerous different hybridization conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Generally, for hybridizations with DNA oligonucleotides without formamide, a temperature of 68 DEG C, and for hybridization with formamide, 50% (v/v), a temperature of 42 DEG C is recommended. For hybridizations with oligonucleotides, the optimal conditions (formamide concentration and/or temperature) depend on the length and base composition of the probe and must be determined individually. In general, optimal hybridization for oligonucleotides of about 10 to 50 bases in length occurs approximately 5 DEG C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched sequences to hybridize and can therefor result in reduced specificity. When using

RNA oligonucleotides with formamide (50% v/v) it is recommend to use a hybridization temperature of 68 DEG C for detection of target RNA and of 50 DEG C for detection of target DNA. Alternatively, a high SDS hybridization solution can be utilized (Church et al. 1984, Proc Natl Acad Sci USA 81:1991-1995). The specificity of hybridization can furthermore be ensured through the presence of a crosslinking moiety on the oligonucleotide (e.g. Huan et al. 2000, Biotechniques 28: 254-255; WO00/14281). Said crosslinking moiety enables covalent linking of the oligonucleotide with the target nucleotide sequence and hence allows stringent washing conditions. Such a crosslinking oligonucleotide can furthermore comprise another label suitable for detection/quantification of the oligonucleotide hybridized to the target.

Detection of PCR amplification can be conventionally performed "in bulk", or with digital PCR (dPCR or dePCR) by means of a multitude of distinct measurements (of e.g. fluorescence). In droplet digital PCR (ddPCR), the reaction mixture is divided in droplets and the signal is measured for each droplet using a droplet flow cytometer.

RPKM (Reads Per Kilobase Million) is often used as measure for expression. FPKM (Fragments Per Kilobase Million) is very similar to RPKM; whereas RPKM was designed for single-end RNA-seq (every read corresponded to a single sequenced fragment), FPKM was designed for paired-end RNA-seq. With paired-end RNA-seq, two reads can correspond to a single fragment, or, if one read in the pair did not map, one read can correspond to a single fragment. The only difference between RPKM and FPKM is that FPKM takes into account that two reads can map to one fragment (and so it doesn't count this fragment twice). When using RNA-seq, reporting or results often is in RPKM (Reads Per Kilobase Million) or FPKM (Fragments Per Kilobase Million). Whatever metric used (another alternative for example is TPM (Transcripts Per Kilobase Million)), such metric is attempting to normalize for sequencing depth and gene length and provide a measure for quantifying transcript levels/gene expression/expression units.

Protein expression level

Determining the level of expression of a protein generally involves use of a labeled ligand capable of binding to the protein. The label can be of enzymatic, fluorescent, isotopic, etc. nature. Such labeled ligand can for instance be a labeled antibody (selectively or specifically) binding to the protein. Protein expression can be measured/quantified on intact cells, in the protein fraction of a sample, or in the protein fraction isolated from isolated cells. Methods of measuring protein levels include western blotting, ELISA, flow cytometry, FACS analysis, etc. Other methods such as proteomics, chromatograph, etc. do not necessarily require a labeled ligand for detection/quantification of a protein of interest.

Inactivation of a process as envisaged in the current invention refers to different possible levels of inactivation, e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or even 100% or more if inactivation (compared to a normal situation). The nature of the inactivating compound is not vital/essential to the invention as long as the process envisaged is inactivated such as to treat or inhibit tumor growth or such as to inhibit relapse of tumor growth.

Pharmacological inhibition of a target of interest

Pharmacological inhibition in general occurs by means of an agent inhibiting at least one of the biological activities (if more than one is known) of a target protein of interest. In particular, such pharmacological inhibitor is binding, such as specifically and/or exclusively binding to a target protein or protein of interest, or is specifically and/or exclusively inhibiting the targeted biological activity of the a target protein of interest.

Such binding may occur with high affinity although this is not an absolute requirement. The pharmacological inhibitor of a target protein or protein of interest may for instance have a binding affinity (dissociation constant) to (one of) its target of about 1000 nM or less, a binding affinity of about 100 nM or less, a binding affinity of about 50 nM or less, a binding affinity of about 10 nM or less, or a binding affinity of about 1 nM or less. Cross-reactivity of a pharmacological inhibitor with more than one protein is possible; for clinical development it can e.g. be desired to be able to test a pharmacological inhibitor in a suitable in vitro or in vivo animal model before starting clinical testing with the same pharmacological inhibitor in a human population, which requires the pharmacological inhibitor to cross-react with the animal (or other non-human) target protein and with the orthologous human target protein (orthologous proteins are homologous proteins separated by a speciation event).

Specificity of binding refers to the situation in which a pharmacological inhibitor is, at a certain concentration (sufficient to inhibit the target protein or protein of interest) binding to the target protein with higher affinity (e.g. at least 2-fold, 5-fold, or at least 10-fold higher affinity, e.g. at least 20-, 50- or 100-fold or more higher affinity) than the affinity with which it is possibly (if at all) binding to other proteins (proteins not of interest). Such specificity of binding is in particular determined within the setting of the target subject (e.g. human patient, or animal model) and thus can encompass/does not exclude binding to (at least one) orthologous target proteins. Exclusivity of binding refers to the situation in which a pharmacological inhibitor is binding only to the target protein of interest (and possibly to (at least one) orthologous target protein).

Alternatively, the pharmacological inhibitor may exert the desired level of inhibition of the targeted biological activity or biological activity of interest of a target protein or protein of interest with an IC50

of 1000 nM or less, with an IC50 of 500 nM or less, with an IC50 of 100 nM or less, with an IC50 of 50 nM or less, with an IC50 of 10 nM or less, or with an IC50 of 1 nM or less.

Cross-inhibition by a pharmacological inhibitor of more than one protein is possible; for clinical development it can e.g. be desired to be able to test a pharmacological inhibitor in a suitable in vitro or in vivo animal model before starting clinical testing with the same pharmacological inhibitor in a human population, which requires the pharmacological inhibitor to cross-inhibit the animal (or other non-human) target protein and the orthologous human target protein.

Specificity of inhibition refers to the situation in which a pharmacological inhibitor is, at a certain concentration (sufficient to inhibit the target protein or protein of interest) inhibiting the target protein with higher efficacy (e.g. with an at least 2-fold, 5-fold, or 10-fold lower IC50, e.g. at least 20-, 50- or 100-fold or more lower IC50) than the efficacy with which it is possibly (if at all) inhibiting other proteins (proteins not of interest). Such specificity of inhibition is in particular determined within the setting of the target subject (e.g. human patient, or animal model) and thus can encompass/does not exclude inhibition of (at least one) orthologous target proteins. Exclusivity of inhibition refers to the situation in which a pharmacological inhibitor is inhibiting only the target protein of interest (or (at least one) orthologous target protein).

Specificity of inhibition may refer to inhibition of a single biological activity of a protein of interest (and possibly of (at least one) orthologue) if the protein of interest is known to have more than one biological activity; or may refer to inhibition of the protein of interest (and possibly of (at least one) orthologue) as such, independent of it possibly having multiple biological activities.

Exclusivity of inhibition refers to the situation in which a pharmacological inhibitor is inhibiting only a single biological activity of a protein of interest (and possibly of (at least one) orthologue) if the protein of interest is known to have more than one biological activity; or may refer to inhibition of only the protein of interest (and possibly of (at least one) orthologue) as such, independent of it possibly having multiple biological activities.

In general, the agent inhibiting a target protein or protein of interest is a polypeptide, a polypeptidic agent, an aptamer, or a combination of any of the foregoing. Examples of such pharmacologic inhibitors, all specifically and/or exclusively binding to and/or inhibiting the target protein of interest include immunoglobulin variable domains, antibodies (in particular monoclonal antibodies) or a fragment thereof, alpha-bodies, nanobodies, intrabodies, aptamers, DARPins, affibodies, affitins, anticalins, monobodies, and bicyclic peptides.

The term “antibody” as used herein, refers to an immunoglobulin (Ig) molecule, which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are

typically tetramers of immunoglobulin molecules. The term "immunoglobulin domain" as used herein refers to a globular region of an antibody chain (such as e.g., a chain of a conventional 4-chain antibody or a chain of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region/immunoglobulin domain. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a two-layer sandwich of about seven antiparallel β-strands arranged in two β-sheets, optionally stabilized by a conserved disulphide bond.

The specificity of an antibody/immunoglobulin/immunoglobulin domain/immunoglobulin variable domain (IVD) for an antigen is defined by the composition of the antigen-binding domains in the antibody/immunoglobulin/IVD (usually one or more of the CDRs, the particular amino acids of the antibody/immunoglobulin/IVD interacting with the antigen, and forming the paratope or antigen-binding site) and the composition of the antigen (the parts of the antigen interacting with the antibody/immunoglobulin/IVD and forming the epitope or antibody binding site). Specificity of binding is understood to refer to a binding between an antibody/immunoglobulin/IVD with a single target molecule or with a limited number of target molecules that (happen to) share an epitope recognized by the antibody/immunoglobulin/IVD.

Affinity of an antibody/immunoglobulin/IVD for its target is a measure for the strength of interaction between an epitope on the target (antigen) and an epitope/antigen binding site in the antibody/immunoglobulin/IVD. It can be defined as:

$$K_A = \frac{[Ab\text{-}Ag]}{[Ab]\,[Ag]}$$

Wherein KA is the affinity constant, [Ab] is the molar concentration of unoccupied binding sites on the antibody/immunoglobulin/IVD, [Ag] is the molar concentration of unoccupied binding sites on the antigen, and [Ab-Ag] is the molar concentration of the antibody-antigen complex. Avidity provides information on the overall strength of an antibody/immunoglobulin/IVD-antigen complex, and generally depends on the above-described affinity, the valency of antibody/immunoglobulin/IVD and of antigen, and the structural interaction of the binding partners.

The term "immunoglobulin variable domain" (abbreviated as "IVD") as used herein means an immunoglobulin domain essentially consisting of four "framework regions" which are referred to in the art and herein below as "framework region 1" or "FR1"; as "framework region 2" or "FR2"; as "framework region 3" or "FR3"; and as "framework region 4" or "FR4", respectively; which framework regions are interrupted by three "complementarity determining regions" or "CDRs", which are referred to in the art and herein below as "complementarity determining region 1" or "CDR1"; as "complementarity determining region 2" or "CDR2"; and as "complementarity determining region 3" or

"CDR3", respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. It is the immunoglobulin variable domain(s) (IVDs) that confer specificity to an antibody for the antigen by carrying the antigen-binding site. Methods for delineating/confining a CDR in an antibody/immunoglobulin/immunoglobulin domain/IVD have been described in the art and include the Kabat, Chothia, IMTG, Martin, Gelfand, and Honneger systems (see Dondelinger et al. 2018, Front Immunol 9:2278).

The term "immunoglobulin single variable domain" (abbreviated as "ISVD"), equivalent to the term "single variable domain", defines molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from "conventional" immunoglobulins or their fragments, wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (VH) and a light chain variable domain (VL) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both VH and VL will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation. In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')2 fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associated) immunoglobulin domains such as light and heavy chain variable domains, i.e., by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen. In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single VH/VHH or VL domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs. As such, the single variable domain may be a light chain variable domain sequence (e.g., a VL-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a VH-sequence or VHH sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit). In one embodiment of the invention, the immunoglobulin single variable domains are heavy chain variable domain sequences (e.g., a VH-sequence); more specifically, the immunoglobulin single variable domains can be heavy chain variable domain sequences that are

derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody. For example, the immunoglobulin single variable domain may be a (single) domain antibody (or an amino acid sequence that is suitable for use as a (single) domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a Nanobody® (as defined herein, and including but not limited to a VHH); other single variable domains, or any suitable fragment of any one thereof. In particular, the immunoglobulin single variable domain may be a Nanobody® (as defined herein) or a suitable fragment thereof. *Note:* Nanobody®, Nanobodies® and Nanoclone® are registered trademarks of Ablynx (now part of Sanofi). For a general description of Nanobodies®, reference is made to the further description below, as well as to e.g. WO2008/020079.

"VHH domains", also known as VHHs, VHH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (i.e., of "antibodies devoid of light chains"; Hamers-Casterman et al. 1993, Nature 363:446-448). The term "VHH domain" has been chosen to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "VL domains"). For a further description of VHHs and Nanobody®, reference is made to the review article by Muyldermans 2001 (Rev Mol Biotechnol 74:277-302), as well as to the following patent applications, which are mentioned as general background art: WO 94/04678, WO 95/04079, WO 96/34103; WO 94/25591, WO 99/37681, WO 00/40968, WO 00/43507, WO 00/65057, WO 01/40310, WO 01/44301, EP 1134231 and WO 02/48193; WO 97/49805, WO 01/21817, WO 03/035694, WO 03/054016 and WO 03/055527; WO 03/050531; WO 01/90190; WO 03/025020; WO 04/041867, WO 04/041862, WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858, WO 06/40153, WO 06/079372, WO 06/122786, WO 06/122787 and WO 06/122825. As described in these references, Nanobody® (in particular VHH sequences and partially humanized Nanobody®) can in particular be characterized by the presence of one or more "hallmark residues" in one or more of the framework sequences. A further description of the Nanobody®, including humanization and/or camelization of Nanobody®, as well as other modifications, parts or fragments, derivatives or "Nanobody® fusions", multivalent constructs (including some non-limiting examples of linker sequences) and different modifications to increase the half-life of the Nanobody® and their preparations can be found in e.g. WO 08/101985 and WO 08/142164.

"Domain antibodies", also known as "Dabs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in e.g., EP 0368684, Ward et al. 1989 (Nature 341:544-546), Holt et al. 2003 (Trends in Biotechnology 21:484-490) and WO 03/002609, WO 04/068820, WO 06/030220, and WO 06/003388. Domain antibodies essentially

correspond to the VH or VL domains of non-camelid mammalians, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, i.e., without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences. Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutic use in humans. It should also be noted that single variable domains can be derived from certain species of shark (for example, the so-called "IgNAR domains", see e.g. WO 05/18629).

When an Fc-region is present in an antibody (any format; the Fc-region either naturally present or introduced by means of genetic engineering), antibody-dependent cellular cytotoxicity (ADCC) can be part of the antibody's action in that when the Fc-region is capable of binding to an Fcγ receptor (FcγR or FCGR) on the surface of an immune effector cell, the cell carrying the antibody's target can be killed or destroyed. When an antibody comprises a (naturally occurring or engineered) C1q binding site, complement-dependent cytotoxicity (CDC) can be part of the antibody's action. When an antibody comprises a (naturally occurring or engineered) Fc domain capable of binding to a specific receptor on phagocytic cells, antibody-dependent cellular phagocytosis (ADCP) can be part of the antibody's action. ADCC-, CDC-, and ADCP-inducing antibodies thus are included herein as means of effectuating pharmacological inhibition of a target of interest.

Alphabodies are also known as Cell-Penetrating Alphabodies and are small 10 kDa proteins engineered to bind to a variety of antigens.

Aptamers have been selected against small molecules, toxins, peptides, proteins, viruses, bacteria, and even against whole cells. DNA/RNA/XNA aptamers are single stranded oligonucleotides and are typically around 15-60 nucleotides in length, although longer sequences of 220nt have been selected; they can contain non-natural nucleotides (XNA) as described for antisense RNA. A nucleotide aptamer binding to the vascular endothelial growth factor (VEGF) was approved by FDA for treatment of macular degeneration. Variants of RNA aptamers are spiegelmers are composed entirely of an unnatural L-ribonucleic acid backbone. A Spiegelmer of the same sequence has the same binding properties of the corresponding RNA aptamer, except it binds to the mirror image of its target molecule.

Peptide aptamers consist of one (or more) short variable peptide domains, attached at both ends to a protein scaffold, e.g. the Affimer scaffold based on the cystatin protein fold. Although not called aptamers, a type of further variation is described in e.g. WO 2004/077062 wherein e.g. 2 peptide loops are attached to an organic scaffold to arrive at a bicyclic peptide (which can be further multimerized). Phage-display screening of such bicyclic peptides to arrive at species binding with high-affinity to a target has proven to be possible in e.g. WO 2009/098450.

DARPins stands for designed ankyrin repeat proteins. DARPin libraries with randomized potential target interaction residues, with diversities of over 10^12 variants, have been generated at the DNA level. From these, DARPins can be selected for binding to a target of choice with picomolar affinity and specificity.

Affitins, or nanofitins, are artificial proteins structurally derived from the DNA binding protein Sac7d, found in *Sulfolobus acidocaldarius*. By randomizing the amino acids on the binding surface of Sac7d and subjecting the resulting protein library to rounds of ribosome display, the affinity can be directed towards various targets, such as peptides, proteins, viruses, and bacteria.

Anticalins are derived from human lipocalins which are a family of naturally binding proteins and mutation of amino acids at the binding site allows for changing the affinity and selectivity towards a target of interest. They have better tissue penetration than antibodies and are stable at temperatures up to 70°C.

Monobodies are synthetic binding proteins that are constructed starting from the fibronectin type III domain (FN3) as a molecular scaffold.

Affibodies are composed of alpha helices and lack disulfide bridges, and are based on the Z or IgG-binding domain scaffold of protein A wherein amino acids located in the parental binding domain are randomized. Screening for affibodies for specific binding to a desired target typically is performed using phage display.

Intrabodies are antibodies binding and/or acting to intracellular target; this typically requires the expression of the antibody within the target cell, which can be accomplished by gene therapy/genetic modification involving introduction in a cell of a suitable genetic construct or vector comprising a suitable promoter (e.g. inducible, organ- or cell-specific,...) operably linked to an intrabody coding sequence.

Pharmacological knock-down of a protein of interest

Several technologies can be applied to cause pharmacological knock-down of a target protein or protein of interest. Outlined hereafter are the general principles of agents causing pharmacological knock-down of a target protein by means of inducing (proteolytic) degradation of that target protein.

A proteolysis targeting chimera, or PROTAC, is a chimeric polypeptidic molecule comprising a moiety recognized by an ubiquitin ligase and a moiety binding to a target protein. Interaction of the PROTAC with the target protein causes it to be poly-ubiquinated followed by proteolytic degradation by a cell's own proteasome. As such, a PROTAC provides the possibility of pharmacologically knocking down a target protein. The moiety binding to a target protein can be a peptide or a small molecule (reviewed in, e.g., Zou et al. 2019, Cell Biochem Funct 37:21-30). Other such target protein degradation inducing technologies include dTAG (degradation tag; see, e.g., Nabet et al. 2018, Nat Chem Biol 14:431), Trim-Away (Clift et al. 2017, Cell 171:1692-1706), chaperone-mediated autophagy targeting (Fan et al. 2014,

Nat Neurosci 17:471–480) and SNIPER (specific and non-genetic inhibitor of apoptosis protein (IAP)-dependent protein erasers; Naito et al. 2019, Drug Discov Today Technol, doi:10.1016/j.ddtec.2018.12.002).

Lysosome targeting chimeras, or LYTACs, are chimeric molecules comprising a moiety binding to a lysosomal targeting receptor (LTR) and a moiety binding to a target protein (such as an antibody). Interaction of the LYTAC with the target protein causes it to be internalized followed by lysosomal degradation. A prototypic LTR is the cation-independent mannose-6-phosphate receptor (ciMPR) and an LTR binding moiety is e.g. an agonist glycopeptide ligand of ciMPR. The target protein can be a secreted protein or a membrane protein (see, e.g., Banik et al. 2019, doi.org/10.26434/chemrxiv.7927061.v1).

The term "inhibitor" of a target as used herein refers to antagonists or inhibitors of function or to antagonists or inhibitors of expression of a target of interest. Antagonists of a target may also be compounds binding to a target (e.g. tumor) cell and causing its killing; examples of such antagonists include e.g. antibody-(cytotoxic) drug-conjugates or antibodies capable of causing ADCC. Interchangeable alternatives for "antagonist" include inhibitor, repressor, suppressor, inactivator, and blocker. An "antagonist" thus refers to a molecule that decreases, blocks, inhibits, abrogates, or interferes with target expression, activation or function.

Downregulating of expression of a gene encoding a target is feasible through gene therapy (e.g., by administering siRNA, shRNA or antisense oligonucleotides to the target gene). Biopharmaceutical and gene therapeutic antagonists include such entities as antisense oligonucleotides, gapmers, siRNA, shRNA, zinc-finger nucleases, meganucleases, TAL effector nucleases, CRISPR-Cas effectors, monoclonal antibodies or fragments thereof, alpha-bodies, nanobodies, intrabodies, aptamers, DARPins, affibodies, affitins, anticalins, monobodies, PROTACs, LYTACs, etc. (general description of these compounds included hereinafter).

Genetic inhibition of a target of interest

Downregulating expression of a gene encoding a target is feasible through gene therapy or gene therapeutic agents, in particular gene therapeutic antagonist agents. Such agents include such entities as antisense oligonucleotides, gapmers, siRNA, shRNA, zinc-finger nucleases, meganucleases, Argonaute, TAL effector nucleases, CRISPR-Cas effectors, and nucleic acid aptamers. In particular, any of these agents is specifically or exclusively acting on or antagonizing the target of interest; or any of these agents is designed for specifically or exclusively acting on or antagonizing the target of interest.

One process of modulating/downregulating expression of a gene/target gene of interest relies on antisense oligonucleotides (ASOs), or variants thereof such as gapmers. An antisense oligonucleotide (ASO) is a short strand of nucleotides and/or nucleotide analogues that hybridizes with the

complementary mRNA in a sequence-specific manner. Formation of the ASO–mRNA complex ultimately results in downregulation of target protein expression (Chan et al. 2006, Clin Exp Pharmacol Physiol 33:533-540; this reference also describes some of the software available for assisting in design of ASOs). Modifications to ASOs can be introduced at one or more levels: phosphate linkage modification (e.g. introduction of one or more of phosphodiester, phosphoramidate or phosphorothioate bonds), sugar modification (e.g. introduction of one or more of LNA (locked nucleic acids), 2'-O-methyl, 2'-O-methoxy-ethyl, 2'-fluoro, S-constrained ethyl or tricyclo-DNA and/or non-ribose modifications (e.g. introduction of one or more of phosphorodiamidate morpholinos or peptide nucleic acids). The introduction of 2'-modifications has been shown to enhance safety and pharmacologic properties of antisense oligonucleotides. Antisense strategies relying on degradation of mRNA by RNase H requires the presence of nucleotides with a free 2'-oxygen, i.e. not all nucleotides in the antisense molecule should be 2'-modified. The gapmer strategy has been developed to this end. A gapmer antisense oligonucleotide consists of a central DNA region (usually a minimum of 7 or 8 nucleotides) with (usually 2 or 3) 2'-modified nucleosides flanking both ends of the central DNA region. This is sufficient for the protection against exonucleases while allowing RNAseH to act on the (2'-modification free) gap region. Antidote strategies are available as demonstrated by administration of an oligonucleotide fully complementary to the antisense oligonucleotide (Crosby et al. 2015, Nucleic Acid Ther 25:297-305).

Another process to modulate expression of a gene/target gene of interest is based on the natural process of RNA interference. It relies on double-stranded RNA (dsRNA) that is cut by an enzyme called Dicer, resulting in double stranded small interfering RNA (siRNA) molecules which are 20-25 nucleotides long. siRNA then binds to the cellular RNA-Induced Silencing Complex (RISC) separating the two strands into the passenger and guide strand. While the passenger strand is degraded, RISC is cleaving mRNA specifically at a site instructed by the guide strand. Destruction of the mRNA prevents production of the protein of interest and the gene is 'silenced'. siRNAs are dsRNAs with 2 nt 3' end overhangs whereas shRNAs are dsRNAs that contains a loop structure that is processed to siRNA. shRNAs are introduced into the nuclei of target cells using a vector (e.g. bacterial or viral) that optionally can stably integrate into the genome . Apart from checking for lack of cross-reactivity with non-target genes, manufacturers of RNAi products provide guidelines for designing siRNA/shRNA. siRNA sequences between 19–29 nt are generally the most effective. Sequences longer than 30 nt can result in nonspecific silencing. Ideal sites to target include AA dinucleotides and the 19 nt 3' of them in the target mRNA sequence. Typically, siRNAs with 3' dUdU or dTdT dinucleotide overhangs are more effective. Other dinucleotide overhangs could maintain activity but GG overhangs should be avoided. Also to be avoided are siRNA designs with a 4–6 poly(T) tract (acting as a termination signal for RNA pol III), and the G/C content is advised to be between 35–55%. shRNAs should comprise sense and antisense sequences (advised to each be 19–21 nt

in length) separated by loop structure, and a 3' AAAA overhang. Effective loop structures are suggested to be 3-9 nt in length. It is suggested to follow the sense-loop-antisense order in designing the shRNA cassette and to avoid 5' overhangs in the shRNA construct. shRNAs are usually transcribed from vectors, e.g. driven by the Pol III U6 promoter or H1 promoter. Vectors allow for inducible shRNA expression, e.g. relying on the Tet-on and Tet-off inducible systems commercially available, or on a modified U6 promoter that is induced by the insect hormone ecdysone. A Cre-Lox recombination system has been used to achieve controlled expression in mice. Synthetic shRNAs can be chemically modified to affect their activity and stability. Plasmid DNA or dsRNA can be delivered to a cell by means of transfection (lipid transfection, cationic polymer-based nanoparticles, lipid or cell-penetrating peptide conjugation) or electroporation. Vectors include viral vectors such as lentiviral, retroviral, adenoviral and adeno-associated viral vectors.

Ribozymes (ribonucleic acid enzymes) are another type of molecules that can be used to modulate expression of a gene/target gene of interest. They are RNA molecules capable of catalyzing specific biochemical reactions, in the current context capable of targeted cleavage of nucleotide sequences, in particular targeted cleavage of a RNA/RNA target of interest. Examples of ribozymes include the hammerhead ribozyme, the Varkud Satellite ribozyme, Leadzyme and the hairpin ribozyme.

Besides the use of the inhibitory RNA technology, modulation of expression of a gene of interest can be achieved at DNA level such as by gene therapy to knock-out, knock-down or disrupt the target gene/gene of interest. As used herein, a "gene knock-out" can be a gene knockdown or the gene can be knocked out, knocked down, disrupted or modified by a mutation such as, a point mutation, an insertion, a deletion, a frameshift, or a missense mutation by techniques such as described hereafter, including, but not limited to, retroviral gene transfer. One way in which genes can be knocked out, knocked down, disrupted or modified is by the use of zinc finger nucleases. Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target a desired DNA sequence/DNA sequence of interest, which enable zinc-finger nucleases to target unique sequence within a complex genome. By taking advantage of the endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms.

Other technologies for genome customization that can be used to specifically or selectively knock out, knock down or disrupt a gene/gene of interest are meganucleases and TAL effector nucleases (TALENs, Cellectis bioresearch). A TALEN® is composed of a TALE DNA binding domain for sequence-specific recognition fused to the catalytic domain of an endonuclease that introduces double strand breaks (DSB). The DNA binding domain of a TALEN® is capable of targeting with high precision a large recognition site (for instance 17bp). Meganucleases are sequence-specific endonucleases, naturally occurring "DNA

scissors", originating from a variety of single-celled organisms such as bacteria, yeast, algae and some plant organelles. Meganucleases have long recognition sites of between 12 and 30 base pairs. The recognition site of natural meganucleases can be modified in order to target native genomic DNA sequences (such as endogenous genes) or DNA sequences of interest. Another recent genome editing technology is the CRISPR/Cas system, which can be used to achieve RNA-guided genome engineering (including knock-out, knock-down or disruption of a gene of interest). CRISPR interference is a genetic technique which allows for sequence-specific control of expression of a gene of interest in prokaryotic and eukaryotic cells. It is based on the bacterial immune system-derived CRISPR (clustered regularly interspaced palindromic repeats) pathway. Recently, it was demonstrated that the CRISPR-Cas editing system can also be used to target RNA. It has been shown that the Class 2 type VI-A CRISPR-Cas effector C2c2 (Cas13a; CRISPR-Cas13a or CRISPR-C2c2) can be programmed to cleave single stranded RNA targets carrying complementary protospacers (Abudayyeh et al. 2016 Science353/science.aaf5573). C2c2 is a single-effector endoRNase mediating ssRNA cleavage once it has been guided by a single crRNA guide toward a target RNA/RNA of interest.

Methods for administering nucleic acids include methods applying non-viral (DNA or RNA) or viral nucleic acids (DNA or RNA viral vectors). Methods for non-viral gene therapy include the injection of naked DNA (circular or linear), electroporation, the gene gun, sonoporation, magnetofection, the use of oligonucleotides, lipoplexes (e.g. complexes of nucleic acid with DOTAP or DOPE or combinations thereof, complexes with other cationic lipids), dendrimers, viral-like particles, inorganic nanoparticles, hydrodynamic delivery, photochemical internalization (Berg et al. 2010, Methods Mol Biol 635:133-145) or combinations thereof.

Many different vectors have been used in human nucleic acid therapy trials and a listing can be found on http://www.abedia.com/wiley/vectors.php. Currently the major groups are adenovirus or adeno-associated virus vectors (in about 21% and 7% of the clinical trials), retrovirus vectors (about 19% of clinical trials), naked or plasmid DNA (about 17% of clinical trials), and lentivirus vectors (about 6% of clinical trials). Combinations are also possible, e.g. naked or plasmid DNA combined with adenovirus, or RNA combined with naked or plasmid DNA to list just a few. Other viruses (e.g. alphaviruses, vaccinia viruses such as vaccinia virus Ankara) are used in nucleic acid therapy and are not excluded in the context of the current invention.

Administration may be aided by specific formulation of the nucleic acid e.g. in liposomes (lipoplexes) or polymersomes (synthetic variants of liposomes), as polyplexes (nucleic acid complexed with polymers), carried on dendrimers, in inorganic (nano)particles (e.g. containing iron oxide in case of magnetofection), or combined with a cell penetrating peptide (CPP) to increase cellular uptake. Organ- or cellular-targeting strategies may also be applied to the nucleic acid (nucleic acid combined with organ- or cell-targeting

moiety); these include passive targeting (mostly achieved by adapted formulation) or active targeting (e.g. by coupling a nucleic acid-comprising nanoparticle with any compound (e.g. an aptamer or antibody or antigen binding molecule) binding to a target organ- or cell-specific antigen) (e.g. Steichen et al. 2013, Eur J Pharm Sci 48:416-427).

CPPs enable translocation of the drug of interest coupled to them across the plasma membrane. CPPs are alternatively termed Protein Transduction Domains (TPDs), usually comprise 30 or less (e.g. 5 to 30, or 5 to 20) amino acids, and usually are rich in basic residues, and are derived from naturally occurring CPPs (usually longer than 20 amino acids), or are the result of modelling or design. A non-limiting selection of CPPs includes the TAT peptide (derived from HIV-1 Tat protein), penetratin (derived from Drosophila Antennapedia – Antp), pVEC (derived from murine vascular endothelial cadherin), signal-sequence based peptides or membrane translocating sequences, model amphipathic peptide (MAP), transportan, MPG, polyarginines; more information on these peptides can be found in Torchilin 2008 (Adv Drug Deliv Rev 60:548-558) and references cited therein. CPPs can be coupled to carriers such as nanoparticles, liposomes, micelles, or generally any hydrophobic particle. Coupling can be by absorption or chemical bonding, such as via a spacer between the CPP and the carrier. To increase target specificity an antibody binding to a target-specific antigen can further be coupled to the carrier (Torchilin 2008, Adv Drug Deliv Rev 60:548-558). CPPs have already been used to deliver payloads as diverse as plasmid DNA, oligonucleotides, siRNA, peptide nucleic acids (PNA), proteins and peptides, small molecules and nanoparticles inside the cell (Stalmans et al. 2013, PloS One 8:e71752).

Any other modification of the DNA or RNA to enhance efficacy of nucleic acid therapy is likewise envisaged to be useful in the context of the applications of a nucleic acid as outlined herein. The enhanced efficacy can reside in enhanced expression, enhanced delivery properties, enhanced stability and the like. The applications of a nucleic acid as outlined herein may thus rely on using a modified nucleic acid as described above. Further modifications of the nucleic acid may include those suppressing inflammatory responses (hypoinflammatory nucleic acids).

Treatment / therapeutically effective amount

"Treatment"/"treating" refers to any rate of reduction, delaying or retardation of the progress of a disease or disorder, or a single symptom thereof, compared to the progress or expected progress of the disease or disorder, or singe symptom thereof, when left untreated. This implies that a therapeutic modality on its own may not result in a complete or partial response (or may even not result in any response), but may, in particular when combined with other therapeutic modalities, contribute to a complete or partial response (e.g. by rendering the disease or disorder more sensitive to therapy). More desirable, the treatment results in no/zero progress of the disease or disorder, or single symptom

thereof (i.e. "inhibition" or “inhibition of progression”), or even in any rate of regression of the already developed disease or disorder, or single symptom thereof. “Suppression/suppressing” can in this context be used as alternative for “treatment/treating”. Treatment/treating also refers to achieving a significant amelioration of one or more clinical symptoms associated with a disease or disorder, or of any single symptom thereof. Depending on the situation, the significant amelioration may be scored quantitatively or qualitatively. Qualitative criteria may e.g. by patient well-being. In the case of quantitative evaluation, the significant amelioration is typically a 10% or more, a 20% or more, a 25% or more, a 30% or more, a 40% or more, a 50% or more, a 60% or more, a 70% or more, a 75% or more, a 80% or more, a 95% or more, or a 100% improvement over the situation prior to treatment. The time-frame over which the improvement is evaluated will depend on the type of criteria/disease observed and can be determined by the person skilled in the art.

A "therapeutically effective amount" refers to an amount of a therapeutic agent to treat or prevent a disease or disorder in a subject (such as a mammal). In the case of tumors or cancers, the therapeutically effective amount of the therapeutic agent is treating a subject having a tumor or cancer, such as to reduce the number of cancer cells; reduce the primary tumor size; inhibit (i.e., slow down to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow down to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, e.g., be measured by assessing the duration of survival (e.g. overall survival), time to disease progression (TTP), response rates (e.g., complete response and partial response, stable disease), length of progression-free survival, duration of response, and/or quality of life.

The term “effective amount” refers to the dosing regimen of the agent (e.g. antagonist as described herein) or composition comprising the agent (e.g. medicament or pharmaceutical composition). The effective amount will generally depend on and/or will need adjustment to the mode of contacting or administration. The effective amount of the agent or composition comprising the agent is the amount required to obtain the desired clinical outcome or therapeutic effect without causing significant or unnecessary toxic effects (often expressed as maximum tolerable dose, MTD). To obtain or maintain the effective amount, the agent or composition comprising the agent may be administered as a single dose or in multiple doses. The effective amount may further vary depending on the severity of the condition that needs to be treated; this may depend on the overall health and physical condition of the subject or patient and usually the treating doctor’s or physician’s assessment will be required to establish what is the effective amount. The effective amount may further be obtained by a combination of different types of contacting or administration.

The aspects and embodiments described above in general may comprise the administration of one or more therapeutic compounds to a subject (such as a mammal) in need thereof, i.e., harboring a tumor, cancer or neoplasm in need of treatment. In general a (therapeutically) effective amount of (a) therapeutic compound(s) is administered to the mammal in need thereof in order to obtain the described clinical response(s).

"Administering" means any mode of contacting that results in interaction between an agent (e.g. a therapeutic compound) or composition comprising the agent (such as a medicament or pharmaceutical composition) and an object (e.g. cell, tissue, organ, body lumen) with which said agent or composition is contacted. The interaction between the agent or composition and the object can occur starting immediately or nearly immediately with the administration of the agent or composition, can occur over an extended time period (starting immediately or nearly immediately with the administration of the agent or composition), or can be delayed relative to the time of administration of the agent or composition. More specifically the "contacting" results in delivering an effective amount of the agent or composition comprising the agent to the object.

Kits

The invention further relates to kits, such a diagnostic kits, comprising tools to detect, determine, measure, assess, quantify or assay the amount of a plexin of class A biomarker. In particular such tools are oligonucleotides capable of detecting, determining, measuring, assessing, assaying or quantifying the amount of nucleic acid corresponding to a plexin of class A; other reagents are, however, not excluded from being part of the kit. Oligonucleotides for instance are primers and/or probes (one or more of them optionally provided on any type of solid support; and one or more of the primers or probes provided may comprise any type of detectable label) specifically targeting (part of) the plexin A nucleic acid . A further reagent part of the kit may be a reagent or reagents for isolating cells such as T-cells (e.g. CD8+ T cells or CTLs), for isolating nucleic acids (from a fluid sample or from cells), or one or more PCR reagents. The kit may also comprise an insert or leaflet, or a computer-readable medium with instructions on how to operate the kit. The kit may further comprise an insert, leaflet, or computer-readable medium with instruction on how to interpret test results obtained by operating the kit. The kit may further comprise a computer-readable medium that causes a computer to compare plexin A biomarker amounts determined by operating the kit to one or more control or reference sample profiles (as discussed above). In an embodiment, the computer readable medium obtains the control or reference sample profiles (as discussed above).

Alternatively, the kit comprises tools to detect, determine, measure, assess, quantify or assay the amount of a plexin of class A biomarker protein, such as a labeled ligand capable of binding to a plexin A protein.

The kit may further comprise tools to detect, determine, measure, assess, quantify or assay the amount of any other biomarker useful for monitoring the response of a subject having a tumor to an anti-tumor therapy, or for determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy.

In particular, such kits find their use in monitoring the response of a subject having a tumor to an anti-tumor therapy, or in determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy by applying any of the methods outlined herein.

Further in particular, such kits are (diagnostic) kits for use in monitoring the response of a subject having a tumor to an anti-tumor therapy, or in determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy, such as by applying any of the methods outlined herein.

Further in particular, such kits are companion diagnostic kits.

The class A plexin biomarker herein described can be used (in any method, use or kit described herein) in combination with other known predictive tissue and/or liquid biomarkers. Predictive tissue biomarkers for immune checkpoint therapy include PD-L1 expression, mismatch repair deficiency (MMR) and/or microsatellite instability (MSI), tumor mutation burden (TMB) and DNA damage response (DDR) alterations or deficiency.

Investigational tissue biomarkers for response to immune checkpoint therapy include expression of one or more of the genes IFN- c, IDO1, CXCL9, CCL4, CCL5, CXCL9, CXCL10, CXCL11, CD8, CD4, CD3, PD-1, FOXP3, LAG3, PD-L2, CTLA-4, Granzyme A, Granzyme B, Perforin-1, CD40, CD27, and HVEM; include detection of alterations in one or more of the genes EGFR, MDM2, MDM4, ALK, EGFR, KRAS/TP53, STK11/LKB1, PBRM1, ATM, POLE, BRCA2, ERCC2, FANCA, MSH6, JAK1, JAK2, B2M, MSH2, PMS2, MLH1, or the IFN-gamma pathway genes; an include determination of the number of tumor-infiltrating lymphocytes (TILs) (see Table 5 of Arora et al. 2019, Adv Ther 36:2638-2678 as well as references referred to therein).

Investigational liquid biomarkers for response to immune checkpoint therapy include lactate dehydrogenase (LDH), Neutrophil-lymphocyte ratio (NLR), absolute eosinophil count, monocyte counts and myeloid derived suppressor cells (MDSCs), T-cell markers, soluble PDL1, B cell-antibody markers, soluble CD25, and blood–tumor mutational burden (bTMB) (see Table 3 of Arora et al. 2019, Adv Ther 36:2638-2678 as well as references referred to therein)

Use

The invention in another aspect relates to the use of a class A plexin biomarker in monitoring the response of a subject having a tumor to an anti-tumor therapy, or in determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy. In particular, such use of a class A plexin biomarker in monitoring the response of a subject having a tumor to an anti-tumor therapy, or in determining, assessing, measuring or predicting the response of a subject having a tumor to an anti-tumor therapy is as explained and described herein, such as in any of the methods outlined herein.

EXAMPLES

EXAMPLE 1. Human PlxnA4 expression in peripheral blood lymphocytes as biomarker for response to immune checkpoint therapy.

Blood samples were collected from patients diagnosed with stage III or stage IV melanoma before (naïve) and after treatment with 1 dose of immune checkpoint inhibition immunotherapy (ICI) with nivolumab (anti-PD1), pembrolizumab (anti-PD1) or the combination of nivolumab (anti-PD1) + ipilimumab (anti-CTLA4) (blood samples were provided by Prof. Dr. Bechter from the Dept. of Dermatology of the university Hospital of Leuven (UZ Leuven)). Patients were treatment naïve for ICI therapy. The on-treatment blood sample was typically collected 3 weeks after the initial blood sample, prior to the second dose of ICI. All donors provided informed consent for the sample collection.

RECIST was used to stratify patients with measurable disease. Most RECIST responses were noticed at first or second radiographic evaluation (i.e. 3-6 months of treatment). Patients with unresectable melanoma received combination therapy of ipilimumab and nivolumab in the neo-adjuvant setting. For these patients without measurable disease stratification was based on pathological response, or a relapse-free interval of one year.

Peripheral blood mononuclear cells (PBMCs) from patients and age-matched healthy volunteers were isolated from whole blood by density gradient centrifugation using Lymphoprep™ (Stemcell). CD8+ T cells were subsequently isolated by negative selection using CD8+ T cell isolation kit (Miltenyi Biotec). Expression of *PLXNA1, PLXNA2, PLXNA3 and PLXNA4* mRNA in isolated circulating CD8+ T cells was determined by quantitative real-time PCR (RT-PCR), and normalized against expression of *TBP* as house-keeping gene. As indicated in Figure 6, a highly significant difference in mRNA levels was observed only for *PLXNA4* and this comparing expression in CD8+ T-cells obtained from healthy individuals *vs* subjects having melanoma. Expression of *PLXNA4* mRNA was also determined in circulating CD4+ T cells and in circulating monocytes; as can be derived from Figure 7, only a slight or no difference in *PLXNA4* mRNA levels was observed in circulating CD4+ T cells and in circulating monocytes, respectively, and this

comparing expression in CD8+ T-cells obtained from healthy individuals *vs* subjects having melanoma or cancer.

Expression of *PLXNA4* mRNA level for paired samples before treatment and on-treatment (indicated as naïve versus treated with ICI) of all patients, confirmed responders and of confirmed non-responders is depicted in Figure 1. Results indicate that *PLXNA44* expression levels in peripheral CD8+ T cells of patients responding to ICI therapy is decreasing, being an early biomarker for response to ICI-therapy. When looking deeper into the responders, the decrease in *PLXNA44* expression levels in peripheral CD8+ T cells in responders appeared more pronounced in responders still eligible for surgical resection of the tumor(s), and less in stage III/IV non-resectable disease stage melanoma patients (not shown). Although a higher number of responders/non-responders is required to reach statistical significance, measuring *PLXNA44* expression levels in peripheral CD8+ T cells may thus be particularly interesting in the neoadjuvant anti-tumor therapy setting.

Quantitative RT-PCR

Total RNA was extracted from sorted CD8+ T cells with TRIzol (Life Technologies) according to the manufacturer's instructions. Reverse transcription of mRNA to cDNA was performed with the SuperScript III First Strand cDNA Synthesis Kit (Life Technologies) according to the manufacturer's instructions. Pre-made assays were purchased from Applied Biosystem. cDNA, primer/probe mix and TaqMan Fast Universal PCR Master Mix were prepared in a volume of 10 µl according to manufacturer's instructions (Applied Biosystems). Samples were loaded into an optical 96-well Fast Thermal Cycling plate (Applied Biosystems) and qRT–PCR were performed using a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Samples were run in technical triplicates.

Data was normalized to a housekeeping gene (HPRT or Tbp) expression. The commercially available probes (Integrated DNA Technologies) used are listed in Table 1.

Table 1. List of probes used for quantitative real-time PCR mRNA expression analysis.

Species	Gene	Exon location	Assay ID
Mouse	*Hprt*	Exon 2-3	Mm.PT.58.32092191
Mouse	*PlxnA4*	Exon 2-3	Mm.PT.58.8104978
Human	*Tbp*	Exon 1-2	Hs.PT.58v.39858774
Human	*PlxnA4*	Exon 29-30	Hs.PT.58.4195119

EXAMPLE 2. PlxnA4 deletion in CD8+ T-cells increases their migratory and proliferative capacities leading to a stronger anti-tumor response

The expression of *Plxna4* in CD8+ T-cells sorted from different organs from healthy and tumor-bearing mice was analyzed by q-PCR. In Figure 2A is shown that *Plxna4* is expressed in circulating CD8+ T-cells

both in healthy and tumor-bearing mice, while its expression is significantly higher in the context of a tumor. No *Plxna4* expression was detected in CD8+ T-cells from lymph nodes (LNs) and spleen in healthy mice (data not shown). In the context of a tumor, *PlxnA4* expression was up-regulated in blood, tumor-draining LNs and in the TME (Figure 2A). *In vitro* T-cell activation experiments showed a robust induction of *Plxna4* expression in CD8+ T-cells after 4 days stimulation with CD3/CD28 (Figure 2B). Together these data suggest an involvement of PlxnA4 in CD8+ T-cells activation in the LNs upon or during antigen presentation.

Since CD8+ T-cells expressed considerable levels of *Plxna4* in the blood of tumor-bearing and healthy mice, a potential role in T cell motility was investigated in *ex vivo* chemotaxis assays using transwell plates. Migration of wildtype and *PlxnA4* knockout CD8+ T-cells was assessed towards CCL21 and CCL19, chemokines involved in T cell homing to the LNs (Girard et al. 2012, Nat Rev Immunol 12:762-773), showing increased migration capacity of *Plxna4*-deficient CD8+ T-cells comparing to their WT counterparts (Figure 2C). Next the migration towards the lymph node was examined in healthy and tumour bearing mice. *Plxna4* KO CD8+ T-cells were more efficient in reaching the LNs upon transfer of WT and *Plxna4* KO CD8+ T-cells into WT mice, as measured by entry into the LNs by flow cytometry and immunohistochemistry (Figure 2D-E, J).

In terms of localization, both WT and *Plxna4* KO CD8+ T-cells were able to enter the paracortical areas of the LNs with no entrapment in the high endothelial venules (HEVs, Figure 2F).

The total number of CD8+ T-cells in the LNs of mice bearing LLC subcutaneous tumors or E0771 orthotopic breast tumors was analyzed by flow cytometry of the tumor-draining LNs. Both *Plxna4* KO mice and *Plxna4* KO→WT chimeras showed increased numbers of $CD8^+$ T cells in the draining LNs comparing to WT mice and WT→WT chimeras, respectively (Figure 2G-H).

The migration capacity of *PlexA4* KO CD8+ T-cells activated with CD3/CD28 was improved compared to activated WT CLTs in *ex vivo* chemotaxis assays towards CXCL9 and CXCL10, chemokines implicated in T cell recruitment to the TME (Figure 2I).

In conclusion, the expression of PlxnA4 on CD8+ T cells appears to negative regulator of CD8+ T-cell migration as loss of PlxnA4 in $CD8^+$ CD8+ T-cells was found to increase their migratory capacity towards the LNs, both in healthy and in tumor conditions.

Furthermore, the homing ability to the tumor of *Plxna4* KO $CD8^+$ T cells also showed to be increased *in vivo*, comparing to WT $CD8^+$ T cells, in a competition assay in mice bearing lung (Figure 2K) and melanoma tumors (Figure 2L).

To assess whether the increased number of *Plxna4* KO CD8+ T-cells in the tumors and in the LNs of tumor-bearing mice was a consequence of the increased migratory capacity of these cells, the *in vitro* proliferation of WT and *Plxna4* KO splenocytes was analyzed in a time-course experiment. In the

presence of primary and co-stimulatory signals (CD3/CD28 activation, respectively) optimized for efficient T-cell activation and expansion, the percentage of $CD8^+$ T cells in the total splenocytes increased over time for both WT and *Plxna4* KO cultures, with. *Plxna4* KO CD8+ T-cells showing increased enrichments as of day 3 (Figure 3A). The proliferation index showed increased proliferation of *Plxna4* KO CD8+ T-cells compared to WT controls at day 4 upon activation (Figure 3B-C). This time-point correlates with the increased expression of *Plxna4* in CD8+ T-cells upon activation (Figure 3F), which may suggest a negative regulation of this protein in CD8+ T-cells proliferation.

In vivo, the expression of the classical early marker of T-cell activation, CD69, showed to be upregulated in CD8+ T-cells in the LNs of KO tumor-bearing mice, compared to their respective WT controls (Figure 3E). Furthermore, intratumoral injections of activated WT and *Plxna4* KO $CD8^+$ T cells, revealed that Plxna4 KO CTLs proliferate significantly more in the tumor bed as compared to WT ones (Figure 3G).

PlxnA4 has a cytoplasmatic region that contains a GTPase activating protein (GAP) domain, which mediates major intracellular signaling through the interaction with small GTPases (Kong et al. 2016, Neuron 91:548-560), and Rac1, a member of the small GTPases family, is necessary for the correct homing of the T cells to the LNs (Faroudi et al. 2010, Blood 116:5536-5547). The regulatory effect of PlxnA4 on the activation of small GTPases in CTLs was checked herein. For that, a GTPase pull down assay was performed to detect GTP-bound Rac1 in both WT and *Plxna4* KO CTLs. *Plxna4*-deficient $CD8^+$ T cells had increased levels of active Rac1 (GTP-bound) when compared with the WT ones. Of note, the levels of GTP-bound Rap1 were indistinguishable between both conditions. Altogether, these data further support the idea that PlxnA4 has a role in controlling CTL motility, via the downstream activity of Rac1 small GTPase. Taken together, these results show that deletion of PlxnA4 in CD8+ T-cells increases their migratory capacity and induces a hyperproliferative response to TCR activation, without affecting their cytotoxicity *in vitro*. *PlxnA4* KO CD8+ T-cells showed an increased activation status in the presence of an antigen, which is the case of draining LNs in tumor-bearing mice, suggesting that PlxnA4 is a negative regulator of CD8+ T-cells in the cancer context.

EXAMPLE 3. PlxnA4-deficient CD8+ T-cells have enhanced anti-tumor efficacy

To evaluate the effect of deletion of PlxnA4 in CD8+ T-cells on the anti-tumor efficacy, the ability of *Plxna4* deficient OT-1 CD8+ T-cells to control the tumor growth of LLC-OVA tumors was verified in an adoptive transfer regimen. Naïve *Plxna4* KO OT-I CD8+ T-cells (CD8-positive T-cells expressing OT-1 and deficient in *Plxna4*), and the respective WT controls were transferred into LLC-OVA tumor-bearing WT recipient mice to monitor tumor progression. The transfer of *Plxna4* KO OT-I CD8+ T-cells lead to a strong abrogation of the normal tumor growth, in comparison to the PBS group (Figure 4A). Wild-type OT-I cells were also able to control tumor growth, but to a significantly lesser extent than the *Plxna4* KO OT-I CD8+

T-cells (Figure 4A). This shows the increased capacity of PlxnA4 KO CD8+ T-cells to migrate towards the LNs and reach the tumor.

In a more therapeutic approach, activated PlxnA4 WT and KO OT-I T cells were adoptively transferred in B16-F10 melanoma tumor-bearing mice. In this setting, adoptive transfer of *Plxna4* KO OT-I CD8+ T-cells likewise was able to control tumor growth to a significantly higher extent than wild-type OT-I CD8+ T-cells, resulting in an increased overall survival of the mice (Figure 4B-E), and an increased number of intratumoral KO OT-I CD8+ T-cells (compared to the number of wild-type OT-I CD8+ T-cells) (Figure 4F). Together these data show that the selective deletion of PlxnA4 in CD8+ T-cells is sufficient to increase anti-tumor immunity in two distinct tumor models, and that targeting PlxnA4 in CD8+ T-cells appears as a valuable strategy to manage several tumor types, including immunologically cold tumors.

EXAMPLE 4. Conditional PlxnA2 deletion in CD8$^+$ T-cells leads to enhanced infiltration of CD8+ T-cells in tumors and to reduced tumor growth

PlexinA2 shares the same ligands and signalling cascade as PlexinA4, and has been reported to be able to form heterodimers with PlexinA4. To assess a potential role of PlexinA2 on CD8+ T-cells in the tumor microenvironment, mRNA expression of plexinA2 was analysed after flow cytometric cell sorting of CD8$^+$ T-cells derived from different tissues from either LLC-tumor bearing mice and healthy mice. Results shown in Figure 5A and 5B indicate that PlxnA2 is highly expressed in circulating CD8$^+$ T cells in healthy and tumor-bearing mice.

To directly decipher the functionality of *Plxn2* expressed on CD8+ T cells in a cancer setting, a conditional knockout model was set-up using the Cre-lox system, PlexinA2 L/L CD8.Cre KO mouse model. Tumor growth was monitored in two distinct syngeneic tumor models, subcutaneous MC38 colon adenocarcinoma (Figure 5C, 5D) and orthotopic E0771 TNBC (Figure 5E, 5F). In both models the *PlxnA2*-specific deletion in CD8$^+$ cells was found to reduce the tumor growth versus the wildtype control group.

The analysis of tumor-infiltrating CD8+ T-cells was done by flow cytometry in orthotopic E0771 tumors grown until day 16. Figure 5G-5H shows the results. The PlxnA2-specific deletion in CD8$^+$ T cells leads to a higher number of CD8$^+$ T cells in blood and primary tumors compared to the WT controls.

In conclusion, the selective loss of PlxnA2 on CD8+ T-cells enhances their tumor infiltration and reduces the tumor growth.

EXAMPLE 5. Bispecific PlxnA4-CD8 antibodies

In order to therapeutically inhibit the PlexinA4 function on immune cells, we generated a panel of PlexinA4-specific VHHs.

To this end, PlexinA4-specific VHHs were isolated from the immune repertoire of llama's that had been immunized with a recombinant human PlexinA4 extracellular domain (ECD) with a C-terminal His6 tag (Cat. Nr. 5856-PA-050, R&D systems) and/or mouse PlexinA4 extracellular domain with a C-terminal His_6 tag (generated in-house, aa 24-1233, Q80UG2.3) using the phage display technology.

Following two selection rounds of immune libraries on biotinylated antigens, screening of individual clones was done in binding ELISA to identify specific binders to human and/or mouse PlexinA4 ECD.

To direct the functional blockade of PlexinA4 towards cytotoxic T cells (CTLs), bispecific VHH constructs were generated in which a PlexinA4-specific VHH was genetically fused to a CD8-binding VHH that is not interfering with CD8 function. Six distinct PlexinA4-specific VHHs with less than 90% overall amino acid sequence identity and substantial differences across the three CDRs) were selected for formatting into bispecific formats with a single human CD8 alpha chain-specific VHH (WO 2019/032661, clone 3CDA5). As reference constructs, each of the PlexinA4 VHHs was also formatted with an irrelevant control VHH. Two PlexinA4 VHHs were in addition formatted with a human CD4-specific VHH (WO 2015/044386, clone 3F11), to be able to confirm specificity towards CD8+ T cells. In each case, the two VHH moieties were genetically linked with a single flexible glycine-serine linker ($[glycine_4\text{-}serine_1]_4$, referred to as 20GS linker), with a C-terminal $Flag_3$-His_6 tag.

Bispecific VHH constructs were introduced in the cDNA3.4 vector for expression in 293F cells, and culture supernatants were purified by HisTrap fast flow affinity chromatography, followed by desalting. Monovalent VHHs were produced in *E. coli* TG-1 strain at 200 mL scale, and VHHs were purified from the periplasmatic extracts by immobilized metal affinity chromatography on Nickel-sepharose (Robocolumn, Repligen), followed by desalting. Protein integrity and purity was confirmed by SDS-PAGE under non-reducing conditions, and quantification was done using Bradford method and Nanodrop. Western blot analysis confirmed the integrity of the flag and His-tags.

By means of biolayer interferometry, the kinetic binding profile determination and of dissociation constants determination of the bispecific VHH constructs in comparison to the monovalent plexinA4 VHH indicated that binding towards human PlexinA4 is preserved in the bispecific formats, with off-rates ranging between 2.2×10^{-3} 1/s and $<1 \times 10^{-6}$ 1/s, essentially following the monovalent PlexinA4 VHHs. Furthermore, simultaneously binding to PlexinA4 and CD8 was confirmed.

Ligand competition exerted by the bispecific VHH molecules essentially follows the competition exerted by the monovalent plexinA4 VHH units, with constructs showing strong Semaphorin6a competition (up to 70% inhibition efficacy), with IC_{50} values ranging between 1.9-2.3 nM; and other constructs being partial inhibitors of Semaphorin 6a interaction (inhibition efficacy between 20-40%).

EXAMPLE 6. Materials and methods

Human samples

Blood samples were collected from metastatic melanoma patients before and after the first cycle of immune checkpoint inhibitor (ICI) therapy (aPD-1 alone or in combination with aCTLA-4). Response assessment of melanoma patients with stage III and IV non-resectable disease was performed as per RECIST1.1 criteria. Patients with complete or partial responses were stratified as responders, while non-responders only achieved stable or progressive disease as best overall response. Patients with resectable stage III disease all underwent complete lymph node dissection, which coincided with the "on-treatment" sampling time point. Pathological response was assessed on the resection specimen. Patients with a complete response were stratified as responders, while patients without pathological complete response were stratified as non-responders. The research using human samples was conducted according to institutional and European Union ethical standards, and all subjects ensured written informed consent to participate in this study.

In brief, peripheral blood mononuclear cells from patients and healthy volunteers were isolated by density gradient centrifugation using Lymphoprep™ (Stemcell). $CD4^+$ and $CD8^+$ T cells were negatively selected using MojoSort™ Human CD4 and CD8 T Cell Isolation Kit (Miltenyi Biotec), according to manufacturer's instructions. For the expression analysis of circulating monocytes, cDNA samples from patients with different tumor types and age-matched healthy controls were used.

Animals

Plxna4 KO mice on a C57BL/6 background were obtained from Dr. Castellani (Institut NeuroMyoGène, Université de Lyon, France). C57BL/6 mice were purchased from Charles River. OT-I mice were purchased from Taconic. All mice were used between 6 and 12 weeks old, without specific gender selection. In all experiments, littermate controls were used. Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven.

Cell lines

Murine Lewis lung carcinoma cells (LLC), B16-F10 melanoma cells, MC38 colon adenocarcinoma, and E0771 medullary breast adenocarcinoma (triple negative breast cancer, TNBC) cells were obtained from the American Type Culture Collection (ATCC). LLC-OVA and B16-F10-OVA cell lines were obtained by viral transduction with a pcDNA3-OVA plasmid. All cells were cultured in DMEM medium supplemented with 10% (heat-inactivated) Fetal Bovine Serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (All Gibco, Thermo Fisher Scientific) at 37 °C in a humidified atmosphere containing 5% CO_2.

Bone marrow transplantation

Six-week-old C56BL/6 recipient mice were lethally irradiated with 9.5 Gy. Subsequently, $1x10^7$ bone marrow cells from the appropriate genotype were injected intravenously (IV) via tail vein. Tumor experiments were initiated 6 to 8 weeks after bone marrow reconstitution. Red and white blood cell count was determined using a hemocytometer on peripheral blood. Also, flow cytometry analysis was carried out in blood collected in heparin with capillary pipettes by retro-orbital bleeding.

Syngeneic tumor models Adherent growing murine cells, $1x10^6$ LLC, $1x10^6$ MC38 and $1x10^5$ B16-F10 or B16-F10 OVA, were injected subcutaneously at the right side of the immunocompetent C57BL/6 mouse in a volume of 200 µl of PBS.. Alternatively, $5x10^5$ E0771 medullary breast adenocarcinoma cell were injected orthotopically in the mammary fat pad of the second nipple on the right side in a volume of 50 µl of PBS. The E0771 model represents an immunologically "cold" breast cancer, and the immune infiltrate is dominated by immunosuppressive mo-MDSCs and M2-type TAMs. C57BL/6 mice bearing syngeneic tumors were randomized into groups (n=5) for treatment when tumor volumes reached $80mm^3$. Tumor volumes were measured three times a week with a caliper and calculated using the formula: $V = \pi \times d^2 \times D/6$, where d is the minor tumor axis and D is the major tumor axis. At the end stage, tumors were weighted and collected for immunofluorescence and/or flow cytometric analyses.

CD8-specific PlexinA2 knockout mice and tumor models

Conditional PlexinA2 (lox/lox) KO mouse line was intercrossed with CD8 specific CD8.CreERT2 mice (constitutive active Cre recombinase), for the specific deletion of *PlxnA2* in CD8+ T cells. Adherent growing 1x10^6 MC38 colon adenocarcinoma cells were injected subcutaneously at the right side of the mouse in a volume of 200 µl of PBS. Tumor volumes were measured three times a week with a caliper and calculated using the formula: V = p x d2 x D/6, where d is the minor tumor axis and D is the major tumor axis. At the end stage, tumors were weighted. 5x10^5 E0771 medullary breast adenocarcinoma cells were injected orthotopically in the mammary fat pad of the second nipple on the right side in a volume of 50 µl of PBS. Tumor volumes were measured three times a week with a caliper and calculated using the formula: V = p x d2 x D/6, where d is the minor tumor axis and D is the major tumor axis. At the end stage, tumors were weighted and collected flow cytometric analyses.

Histology and immunostainings

Tumors and lymph nodes (LNs) were collected and fixed in 2% PFA for 24 hours, washed in 70% ethanol and embedded in paraffin. Serial sections were cut at 7 µm thickness with HM 355S automatic microtome (Thermo Fisher Scientific). Paraffin slides were first rehydrated to further proceed with antigen retrieval in Target Retrieval Solution, Citrate pH 6.1 (DAKO, Agilent). If necessary, 0.3% hydrogen peroxide was added to methanol, to block endogenous peroxidases.

Alternatively, lymph nodes (LNs) were collected in OCT compound (Leica) and frozen at -80 °C. After cryo-sectioning (7 µm thickness), samples were thawed and washed with PBS once, followed by fixation with 4% PFA, for 10 minutes at room temperature. After 3 washed, endogenous peroxidases activity was blocked by incubating the sections in methanol containing 0.3% hydrogen peroxide.The sections were blocked with the appropriate serum (DAKO, Agilent; or e.g., 5% FBS and 5% rat serum) and incubated overnight with the following antibodies: rat anti-F4/80 (CI:A3-1, Serotec) 1:100, rabbit anti-Hypoxyprobe-1-Mab1 (Hypoxyprobe kit, Chemicon) 1:100, rat anti-CD34 (RAM34, BD Biosciences) 1:100, rat anti-CD31 (MEC 13.3, BD Biosciences) 1:50, rabbit anti-NG2 (Millipore) 1:200, rat anti-CD8 (4SM16, Thermo Fisher Scientific) 1:100, rat anti-PNAd (MECA-79, Biolegend) 1:100, or Biotin anti-mouse/human PNAd (MECA-79, Biolegend) 1:100. Hoechst 33342 solution (Thermo Fisher Scientific, 1:1000) was used to stain nuclei. Appropriate secondary antibodies were used: Alexa 488, 647 or 568 conjugated secondary antibodies (Molecular Probes), biotin-labeled antibodies (Jackson Immunoresearch) and, when necessary, TSA Plus Cyanine 3 and Cyanine 5 System amplification (Perkin Elmer, Life Sciences) were performed according to the manufacturer's instructions. Whenever sections were stained in fluorescence, ProLong Gold mounting medium without DAPI (Invitrogen) was used. Microscopic analysis was done with an Olympus BX41 microscope and CellSense imaging software.

Flow cytometry

Tumor-bearing mice were sacrificed by cervical dislocation, and tumors, tumor-draining and non-draining LNs were harvested. Tumors were minced in αMEM medium (Lonza), containing Collagenase V (Sigma), Collagenase D (Roche) and Dispase (Gibco), and incubated in the same solution for 30 minutes at 37°C. The digested tissue was filtered using a 70 µm pore sized mesh and cells were centrifuged 5 minutes at 300 ×g. LNs were processed on a 40 µm pore cell strainer in sterile PBS and cells were centrifuged for 10 minutes at 300 ×g. Blood samples were collected in heparin with capillary pipettes by retro-orbital bleeding. Red blood cell lysis was performed by using Hybri-Max™ (Sigma-Aldrich) or by using a home-made red blood cell lysis buffer (150 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$, pH 7.4). Cells were resuspended in FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and incubated for 15 minutes with Mouse BD Fc Block purified anti-mouse CD16/CD32 mAb (BD-Pharmingen) and stained for 30 minutes at 4 °C with: Fixable viability dye (eFluor™ 450 or eFluor™ 506, 1:500), anti-CD11b (M1/70, eFluor™ 506, 1:400), anti-F4/80 (BM8, Alexa Fluor® 488, 1:200), anti-CD8 (53-6.7, APC or APC-Cy7, 1:400; or Alexa Fluor™ 488, 1:100), anti-CD69 (H1.2F3, APC, 1:200), anti-IFN (XMG1.2, PE-Cy7 1:100), anti-Gata3 (TWAJ, eFluor 660, 1:50), anti-T-bet (4B10, PE-Cy7, 1:40), anti-FOXP3 (FJK-16s, PerCP-Cy5.5, 1:100) or anti-TCR Vβ5.1/5.2 (MR9-4, APC, 1:200)- from Thermo Fisher Scientific; anti-CD45 (30-F11, APC-Cy7, 1:300, or PerCP, 1:200), anti-CD115 (AFS98, PE-Cy7, 1:200), anti-CD4 (RM4-5, PerCP-Cy5.5 or APC-Cy7, 1:400), anti-granzyme B (GB11, Alexa Fluor® 647, 1:100) - from BioLegend; and anti-TCRβ (H57-597,

BV421, 1:300) and anti-Ly-6G (1A8, PE, 1:500) - from BD Biosciences. Cells were subsequently washed and resuspended in FACS buffer before FACS analysis or flow sorting by a FACS Verse, FACS Canto II or FACS Aria III (BD Biosciences), respectively. Data was analyzed by FlowJo (TreeStar).

T cell isolation and activation

Naïve T cells were isolated from spleen, inguinal and axillary LNs. In brief, tissues were processed on a 40 µm pore cell strainer in sterile PBS and cells were centrifuged for 10 minutes at 300 ×g. Red blood cell lysis was performed using Hybri-Max™ (Sigma-Aldrich). Total splenocytes were cultured in T cell medium – RPMI medium supplemented with (heat-inactivated) 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin, 1% MEM Non-Essential Amino Acids (NEAA), 25 µm beta-mercaptoethanol and 1 mM Sodium Pyruvate (all Gibco, Thermo Fisher Scientific) – at 37 °C in a humidified atmosphere containing 5% CO_2. According to the experimental requirements, T cells were activated for 3 days by adding CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a bead-to-cell ratio of 1:1 and 30 U/ml rIL-2 (PeproTech).

At day 3 of activation, the beads were magnetically removed and activated T cells were further expanded for a maximum of 3 additional days in the presence of 30 U/ml rIL-2. To monitor cell proliferation, naïve T cells were labelled with 3.5 µM violet cell tracer (Thermo Fisher Scientific) at 37°C for 20 minutes. The cells were subsequently washed with FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and cultured according to the experimental requirements.

T cell migration assay

Migration of $CD8^+$ cells was assessed by using transwell permeable supports with 5-µm polycarbonate membrane (Costar). $CD8^+$ cells were isolated by using MagniSort Mouse CD8 T cell negative selection kit (eBioscience) according to the manufacturer's instructions. To determine cell migration in response to soluble factors, the lower chamber was pre-incubated with 0,1% FBS, 200 ng/ml CCL21 and 200 ng/ml CCL19 (all Peprotech) in T cell medium. $CD8^+$ cells were incubated for 3 incubated for 3 hours at 37°C and migrated cells were collected and counted under the microscope. Alternatively, to determine cell migration in response to soluble factors, the lower chamber was pre-incubated with 0,1% FBS, 200 ng/ml CCL21, 200 ng/ml CCL19, 150 ng/ml CXCL9 and 50 ng/ml CXCL10 (all Peprotech) in T cell medium. $CD8^+$ T cells were incubated for 2 (activated) or 3 hours (naïve) at 37°C and migrated cells in the bottom chamber were collected and counted by FACS using Precision Count Beads™ (Biolegend).

Western blotting

Protein concentration of cell extracts was determined by using Pierce™ bicinchoninic acid (BCA) reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples containing equivalent amounts of protein were subjected to 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane using the Trans-Blot Turbo™ Transfer System (Bio-Rad)

according to manufacturer's instructions. The membranes were blocked for non-specific binding in 5% non-fatty dry milk in Tris Buffered Saline-Tween 0.1 % (50 mM Tris HCl ph 7.6, 150 mM NaCl, 0.1% Tween; TBS-T) for 1 hour at room temperature (RT) and incubated with primary antibody overnight (ON) at 4°C. The following antibodies were used: mouse anti-Rac1 (1:1000, Thermo Fisher Scientific). After incubation with the primary antibody, the membrane was washed for 15 minutes in TBS-T and incubated with the appropriate secondary antibody (1/5000 in 5% non-fatty dry milk in TBS-T) for 1 hours at RT. The following secondary antibodies were used: goat anti-mouse IgG-HRP (Santa Cruz biotechnology). The signal was visualized with Enhanced Chemiluminescent Reagents (ECL; Invitrogen) or SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) with a digital imager (ImageQuant LAS 4000, GE Health Care Life Science Technologies). The results of the GTPase pull down assay were normalized against the corresponding band of the total proteins and quantified by densitometry.

hours at 37°C and migrated cells were collected and counted under the microscope. Alternatively, to determine cell migration in response to soluble factors, the lower chamber was pre-incubated with 0,1% FBS, 200 ng/ml CCL21, 200 ng/ml CCL19, 150 ng/ml CXCL9 and 50 ng/ml CXCL10 (all Peprotech) in T cell medium. $CD8^+$ T cells were incubated for 2 (activated) or 3 hours (naïve) at 37°C and migrated cells in the bottom chamber were collected and counted by FACS using Precision Count Beads™ (Biolegend).

T cell homing assay

$CD8^+$ T cells were isolated from WT and *Plxna4* KO mice and were labelled with either 3.5 µM violet cell tracer (Thermo Fisher Scientific) or 1 µM carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific). Healthy C57BL/6 mice were injected IV with a 1:1 mixture between 1-2 x 10^6 WT and KO T-cells. After 2 hours, lymph nodes (LNs) of the recipient mice were harvested. LNs were used for immunohistochemistry and flow cytometry to determine the percentage of WT and KO T-cells.

Tumor homing assay

Activated WT and *Plxna4* KO OT-I T cells were labelled with either 3.5 µM of Violet Cell Tracer or 1 µM of CFSE and injected intravenously with a 1:1 mixture between 2-3x10^6 WT and *Plxna4*-deficient OT-I T cells into WT recipient mice with established B16-F10-OVA or LLC-OVA tumors. The tumors of recipient mice were harvested 24 and 48 hours after T cell transfer and analyzed by flow cytometry.

Adoptive Cell Transfer

$CD8^+$ T cells were isolated from transgenic *Plxna4* WT/KO OT-I mice, generated by the intercross of *Plxna4* heterozygous mice with OT-I positive mice in the host lab. These mice have a monoclonal population of naïve TCR transgenic $CD8^+$ T cells (OT-I T cells) that recognize the immunodominant cytosolic chicken ovalbumin (OVA) "SIINFEKL" (SEQ ID NO:1) peptide. 1-2x10^6 WT and *Plxna4* KO OT-I T cells were injected into WT recipient mice carrying subcutaneous LLC-OVA tumors (8x10^5 cells injected 5 days before T cell transfer).

Total splenocytes isolated from OT-1-PlexinA4 KO mice and littermate controls were activated with SIINFEKL (SEQ ID NO:1) peptide in the presence of IL-2. Six days later, $CD8^+$ T cells were inoculated intravenously (2,5x10^6 cells per mouse) into recipient mice carrying subcutaneous B16-OVA tumors (1x10^5 cells injected 13 days before T cell transfer). For activation of OT-I T cells, total splenocytes from OT-I mice were isolated and cultured for 3 days in T cell medium with 1 µg/ml SIINFEKL (SEQ ID NO:1) peptide (IBA - LifeSciences) and 30 U/ml rIL-2 (PeproTech). At day 3 of activation, OT-I T cells were further expanded for a maximum of 3 additional days in the presence of 30 U/ml rIL-2. Recipient mice were treated with cyclophosphamide (100mg/kg) 1 day before receiving effector $CD8^+$ T cells and received daily i.p. injections of 5ug of recombinant human IL-2 beginning the day of adoptive transfer and lasting for 4 days.

WT recipient mice carrying orthotopic B16-F10-OVA tumors (average tumor size of 30-50 mm^3) were injected intravenously with either PBS, 2-3x10^6 WT or the same number of *Plxna4* KO OT-I T cells. Recipient mice received daily intraperitoneal (IP) injections of 5µg of recombinant human IL-2, beginning the day of adoptive transfer and lasting for 4 days. Tumor volume was measured at least 4 times per week and at the end of the experiment tumors were weighted and collected for flow cytometric analysis.

Statistics

Data entry and all analyses were performed in a blinded fashion. All statistical analyses were performed using GraphPad Prism software on mean values, calculated from the averages of technical replicates. Statistical significance was calculated by two-tailed unpaired t-test (or paired t-test in the case of *in vivo* homing assay) on two experimental conditions or two-way ANOVA when repeated measures were compared, with $p < 0.05$ considered statistically significant. Survival curves were compared with the log-rank (Mantel-Cox) test. Statistical details of the experiments can be found in the figure legends. Detection of mathematical outliers was performed using the Grubbs' test in GraphPad. Sample sizes for all experiments were chosen based on previous experiences. Independent experiments were pooled and analyzed together whenever possible. All graphs show mean values ± SEM.

CLAIMS

1. A method for monitoring response of a subject having a tumor to anti-tumor therapy, the method comprising quantifying an amount of biomarker which is a plexin of class A in a first and a second fluid sample;

wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy to the subject, and said first fluid sample is obtained from the subject before or at administration of the at least one dose of the anti-tumor therapy to the subject or said first fluid sample is a control sample; and

wherein a reduction of the amount of the biomarker in said second fluid sample compared to said first fluid sample is predictive for the subject to be responsive to said anti-tumor therapy; and wherein the absence of a reduction of the amount of the biomarker in the second fluid sample compared to the first fluid sample is predictive for the subject not to be responsive to said anti-tumor therapy.

2. The method of claim 1, wherein said biomarker is human plexin A4 (plxnA4).

3. The method of claim 1 or 2, wherein said fluid sample is a sample selected from blood, urine, saliva, or a fraction thereof.

4. The method of any one of the previous claims, wherein said method comprises a step of enriching T-cells from said fluid sample to obtain an enriched sample and quantifying the amount of the biomarker in said enriched sample.

5. The method of any one of the previous claims, wherein quantifying the amount of the biomarker comprises quantifying the amount of a nucleic acid of said biomarker, preferably the amount of RNA of said biomarker.

6. The method of any one of the previous claims, wherein said tumor is a solid tumor or an inflamed tumor.

7. The method of any one of the previous claims, wherein said anti-tumor therapy comprises anti-tumor immunotherapy.

8. The method of any one of the previous claims, wherein said anti-tumor therapy comprises immune checkpoint inhibitory therapy.

9. The method of claim 8, wherein the immune checkpoint inhibitory therapy comprises administration of an inhibitor of PD-1, PD-L1, or CTLA4.

10. A method for monitoring response of a subject having a tumor to an anti-tumor therapy comprising immune checkpoint inhibitory therapy, comprising quantifying the amount of plxnA2 RNA and/or plxnA4 RNA in T-cells enriched from a first and a second fluid sample;
wherein said second fluid sample is obtained from the subject after administration of at least one dose of the anti-tumor therapy to the subject, and said first fluid sample is obtained from the subject before or at administration of the at least one dose of the anti-tumor therapy to the subject, or said first fluid sample is a control or reference sample; and
wherein a reduction of the amount of human plxnA4 RNA in said second fluid sample compared to said first sample is predictive for the subject to be responsive to said anti-tumor therapy; and
wherein the absence of a reduction of the amount of human plxnA4 RNA in the second fluid sample compared to the first fluid sample is predictive for the subject not to be responsive to said anti-tumor therapy.

11. The method of any one of the previous claims wherein said second fluid sample is obtained from the subject two to four weeks after administration of a dose of the anti-tumor therapy.

12. A plexin class A antagonist for use in the treatment of a subject having a tumor, wherein the subject has been indicated to be not responsive to an anti-tumor therapy not comprising a plexin class A antagonist using the method of any one of the previous claims.

13. The plexin class A antagonist for use according to claim 12 wherein the anti-tumor therapy not comprising a plexin class A antagonist is replaced or combined with a second anti-tumor therapy.

14. The plexin class A antagonist for use according to claim 13, wherein said second anti-tumor therapy comprises administration of a plexin class A antagonist.

15. A kit comprising at least one oligonucleotide to quantify the amount of a plexin of class A biomarker for use in a method of any one of the previous claims.

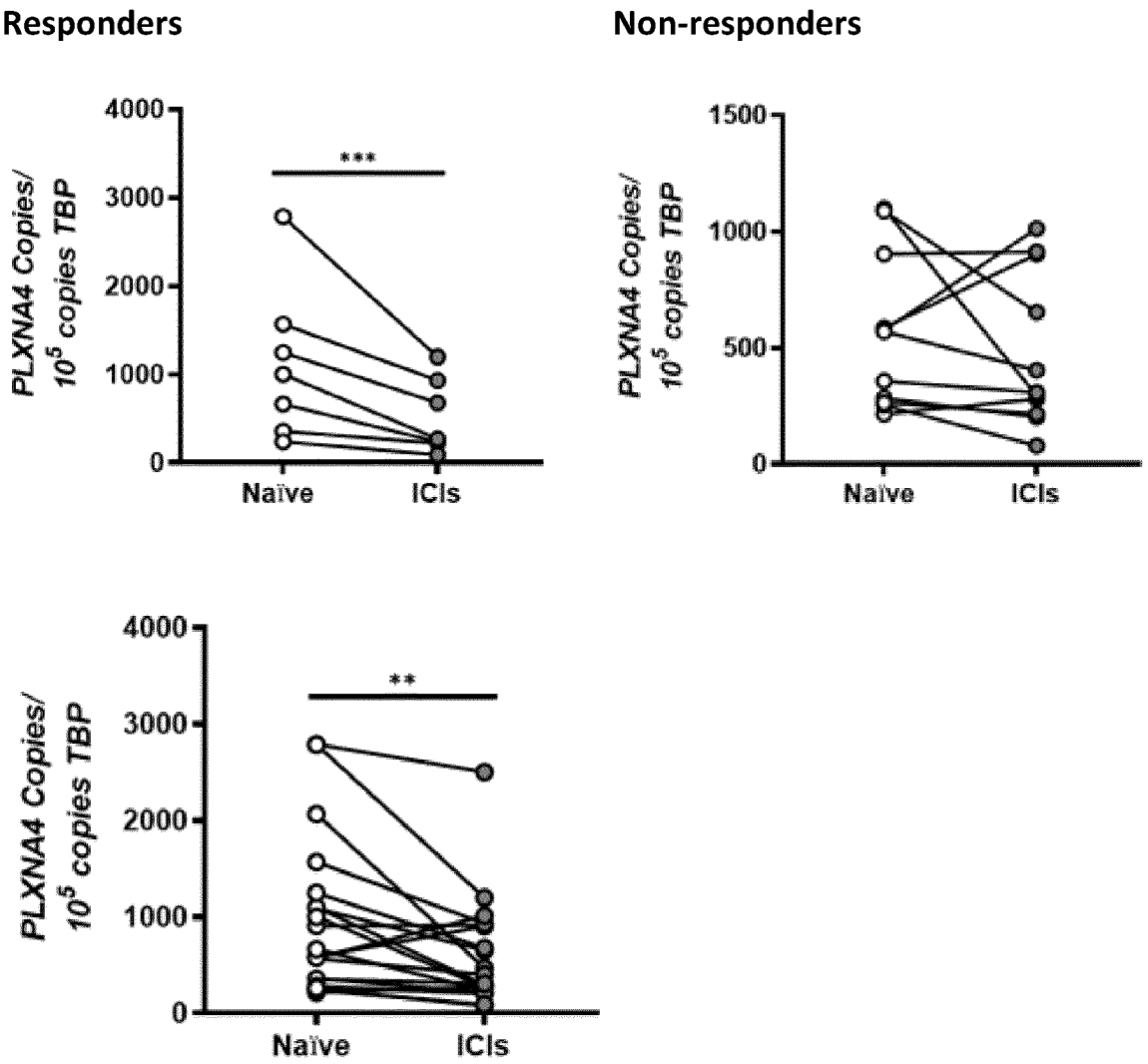


FIGURE 1

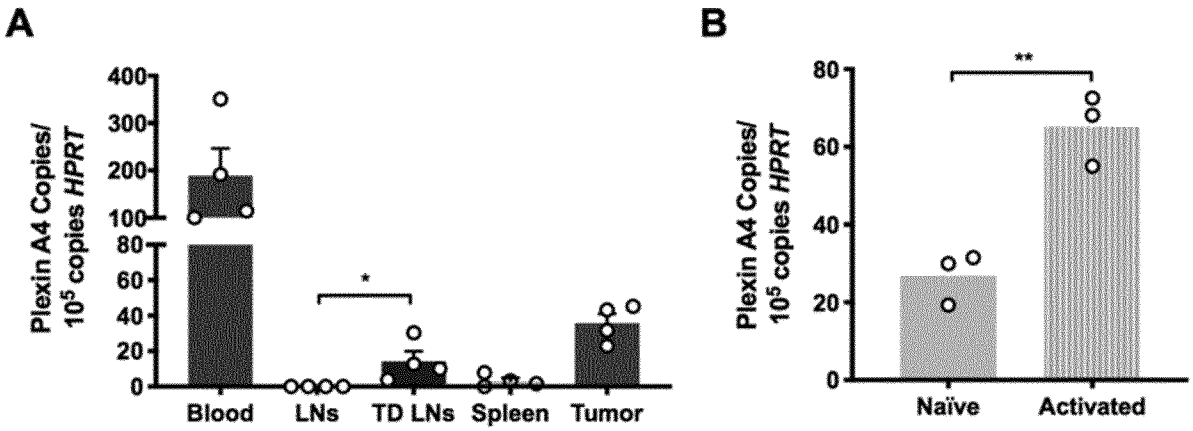


FIGURE 2

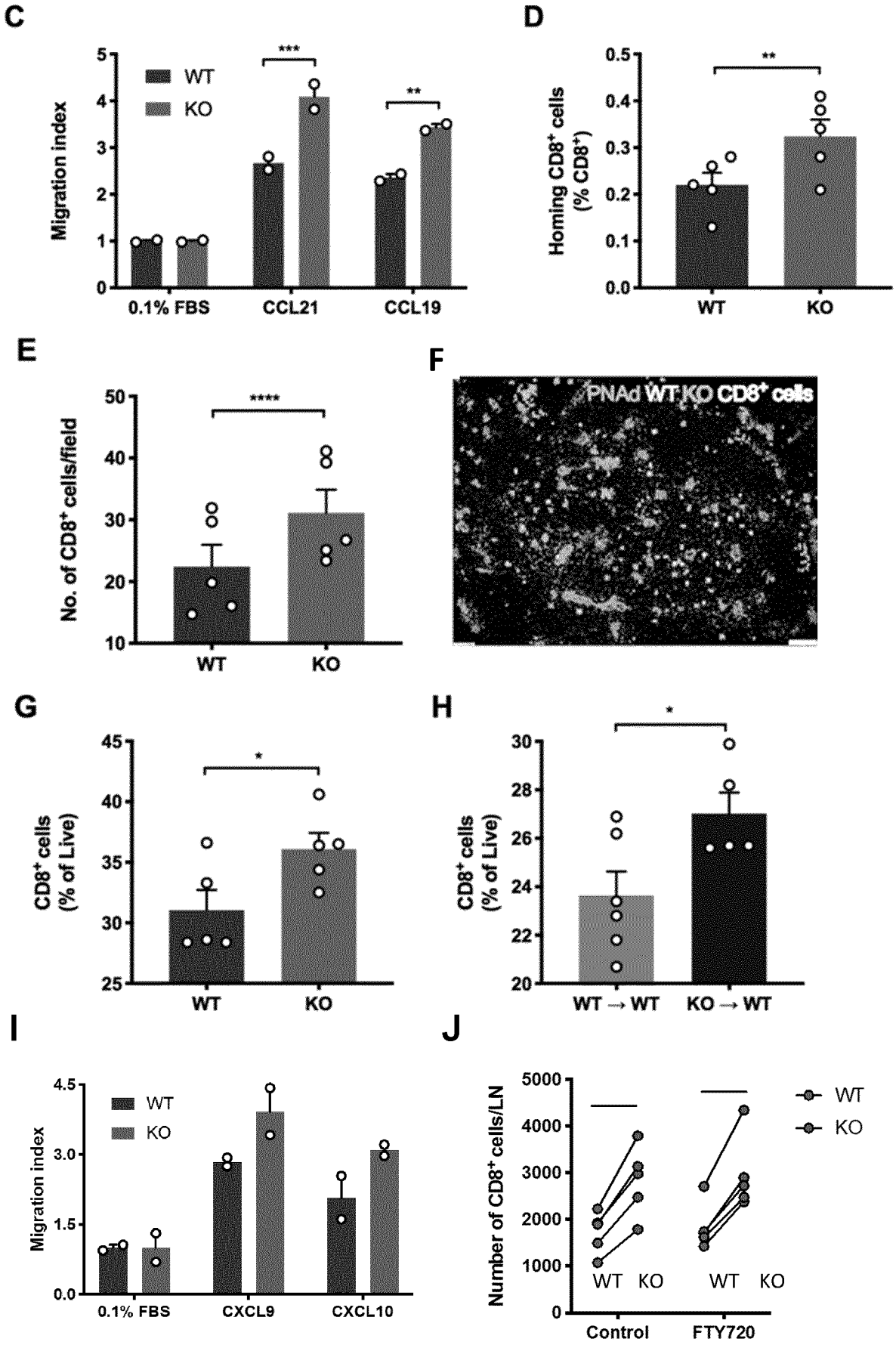


FIGURE 2 - continued

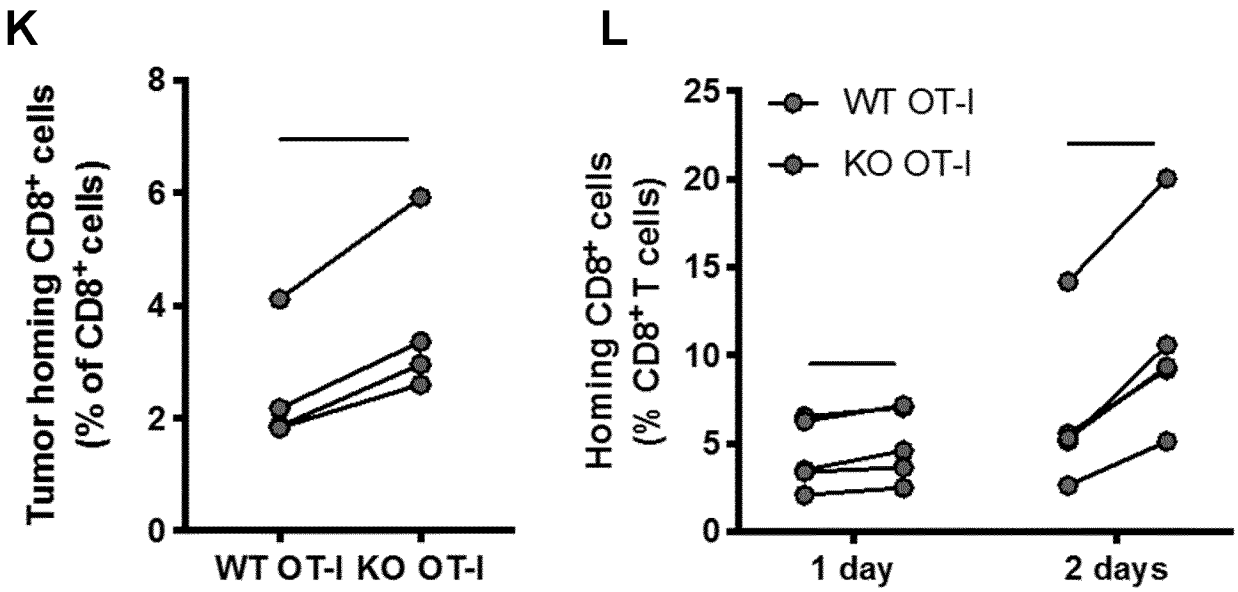


FIGURE 2 - continued

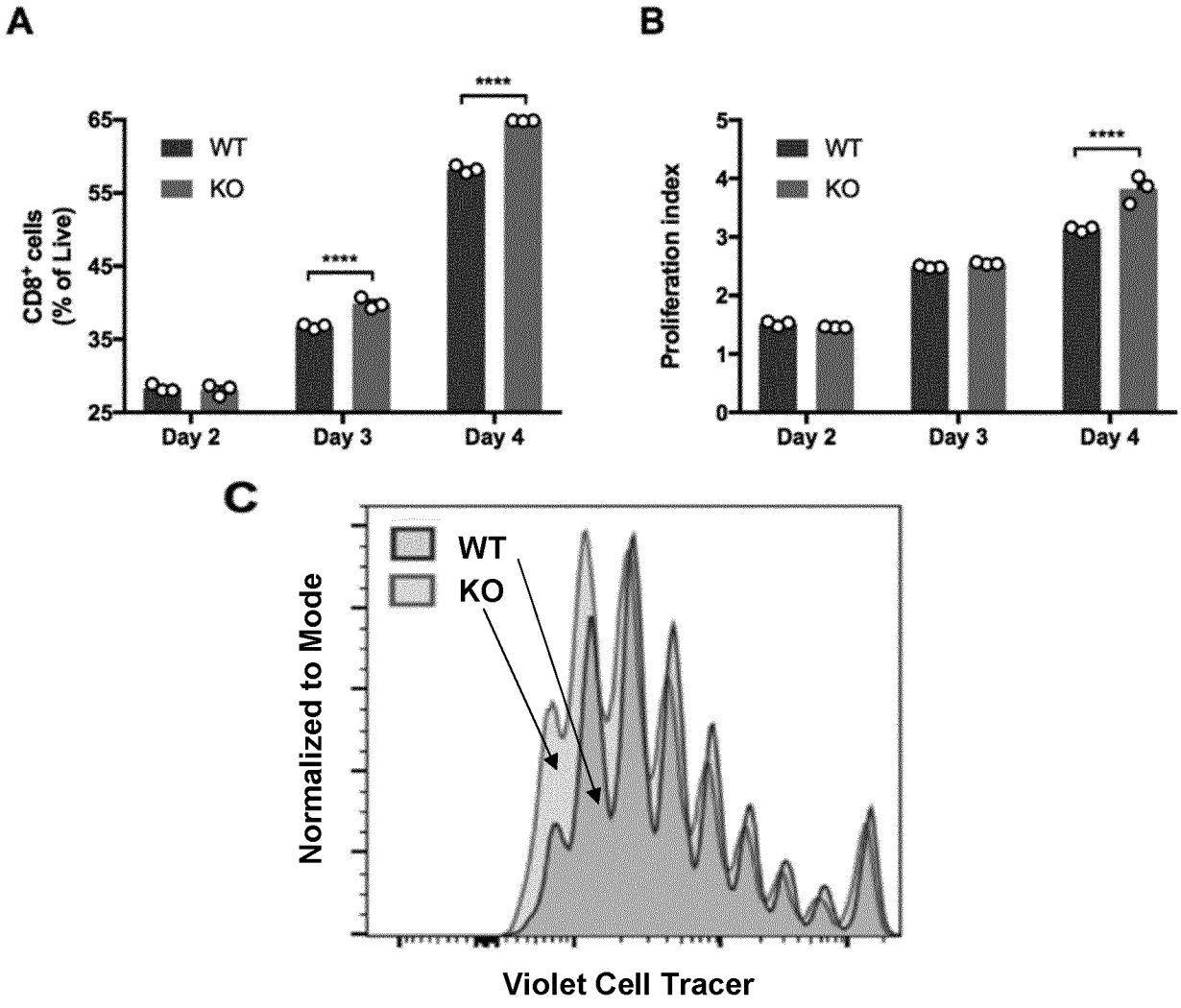


FIGURE 3

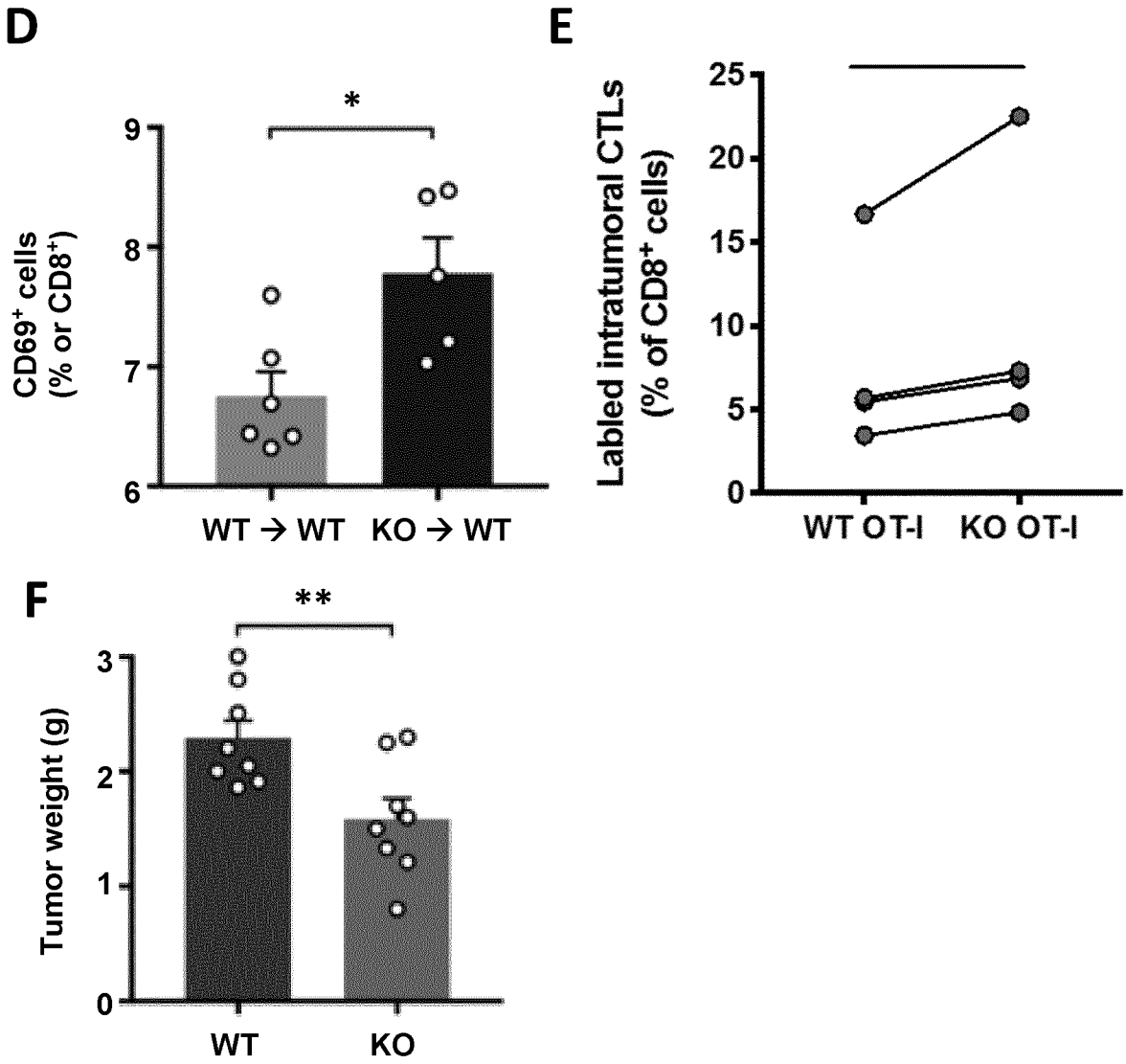


FIGURE 3 - continued

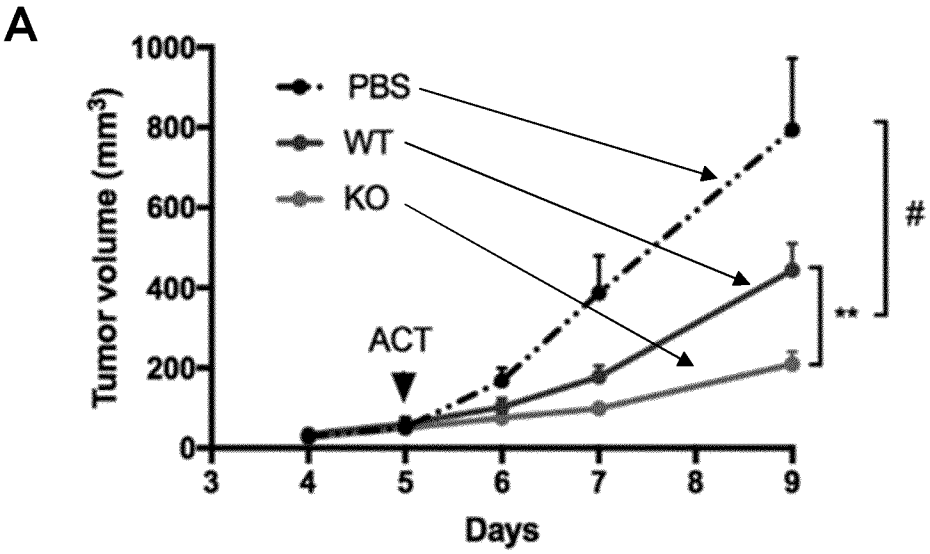


FIGURE 4

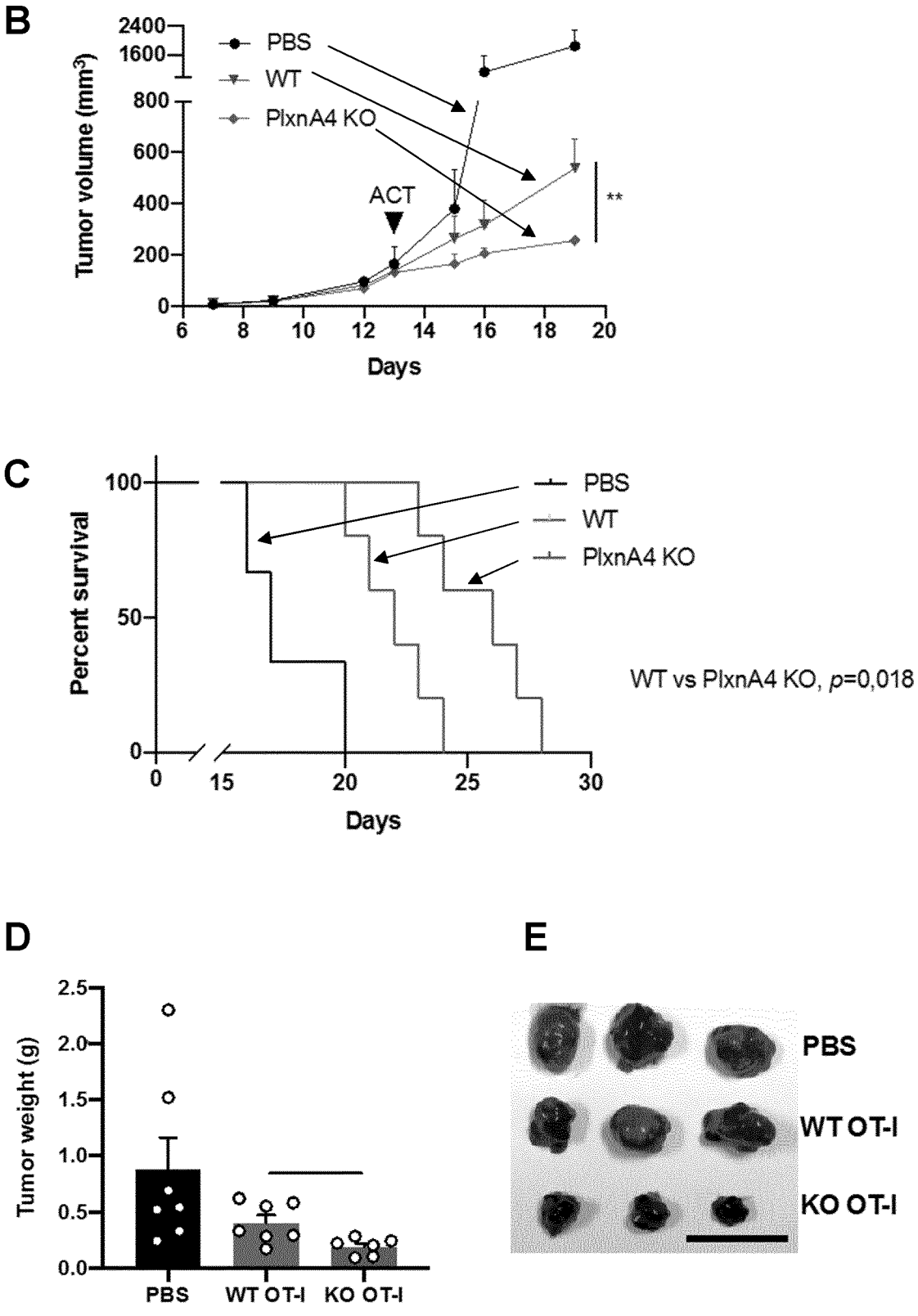
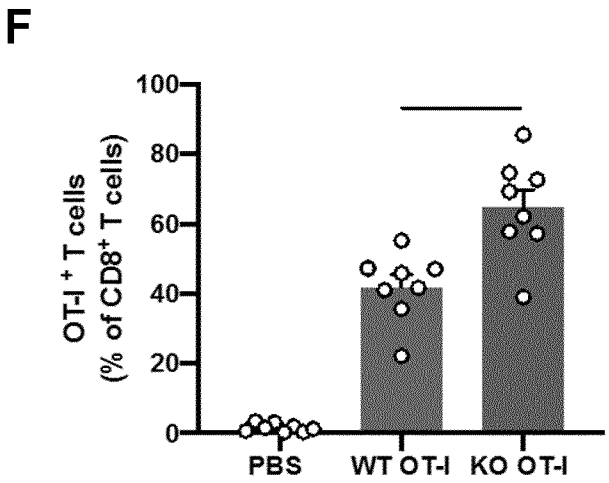


FIGURE 4 - continued



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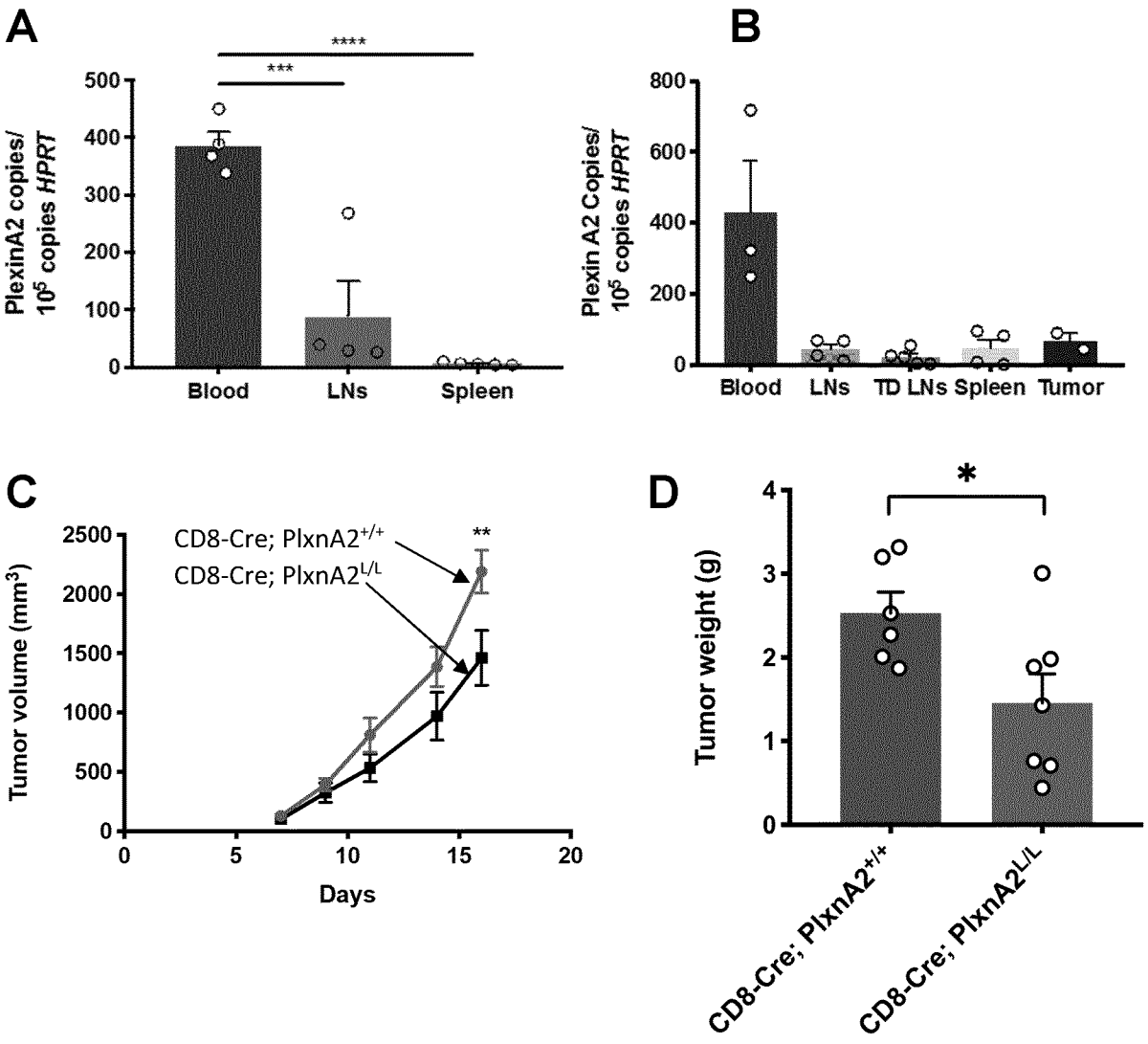

FIGURE 5

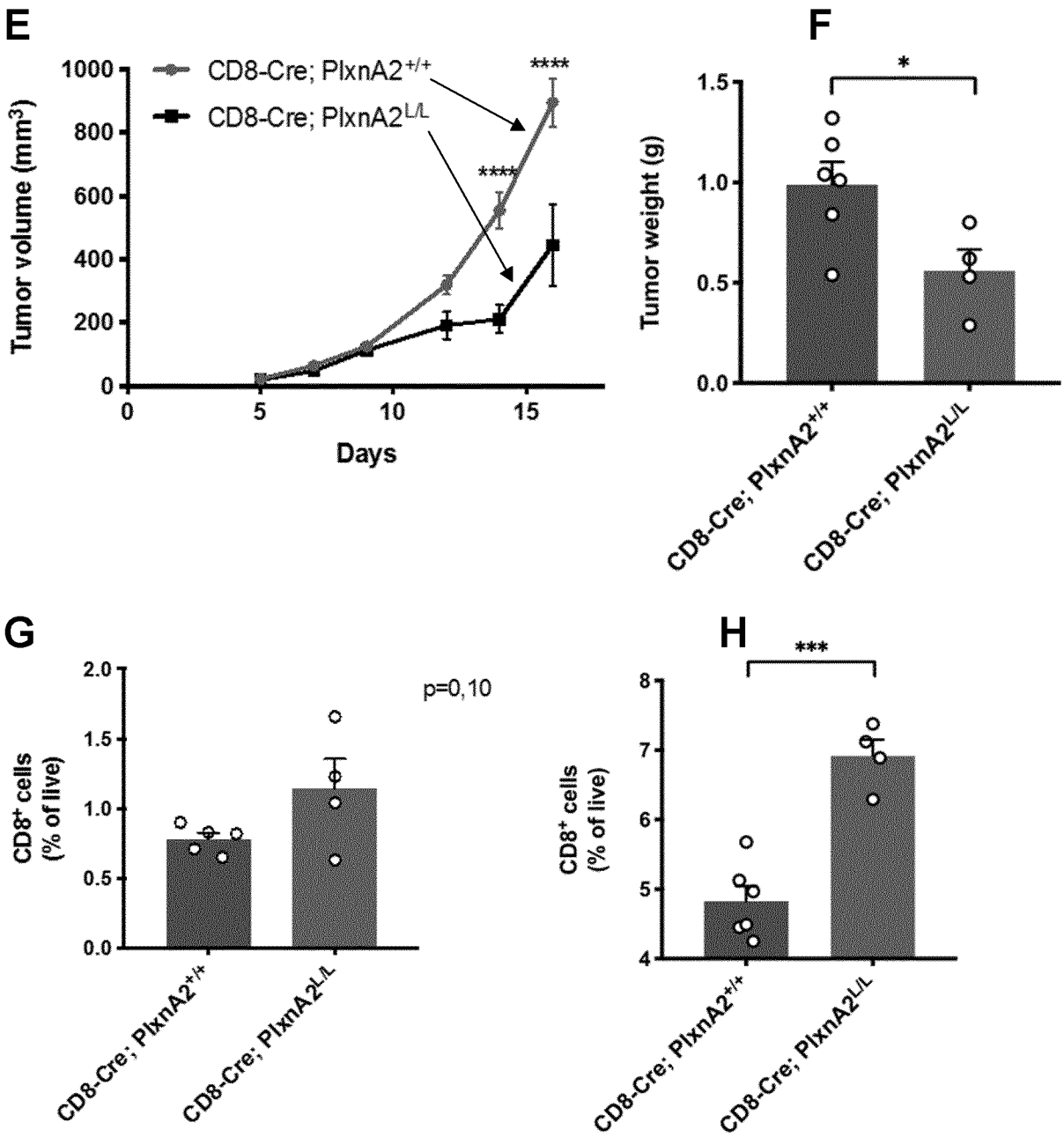

FIGURE 5 - continued

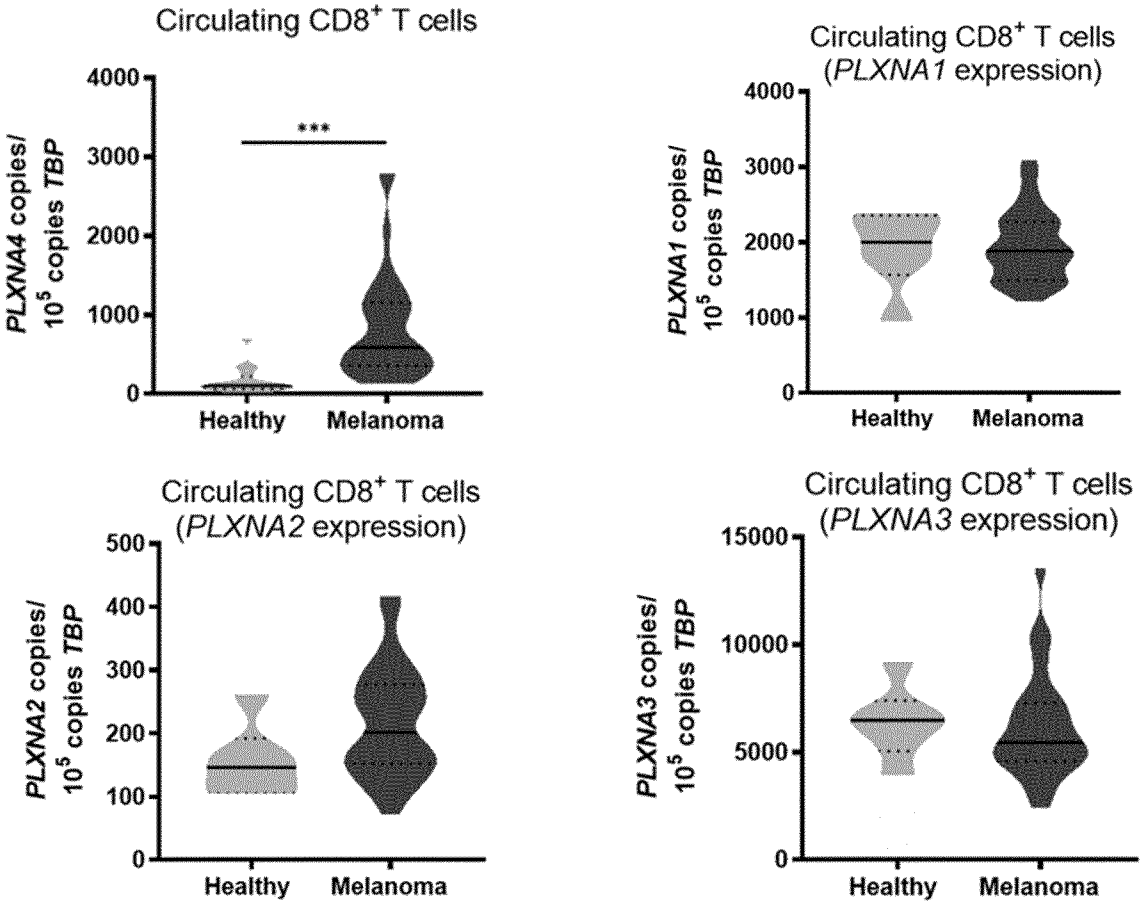

FIGURE 6

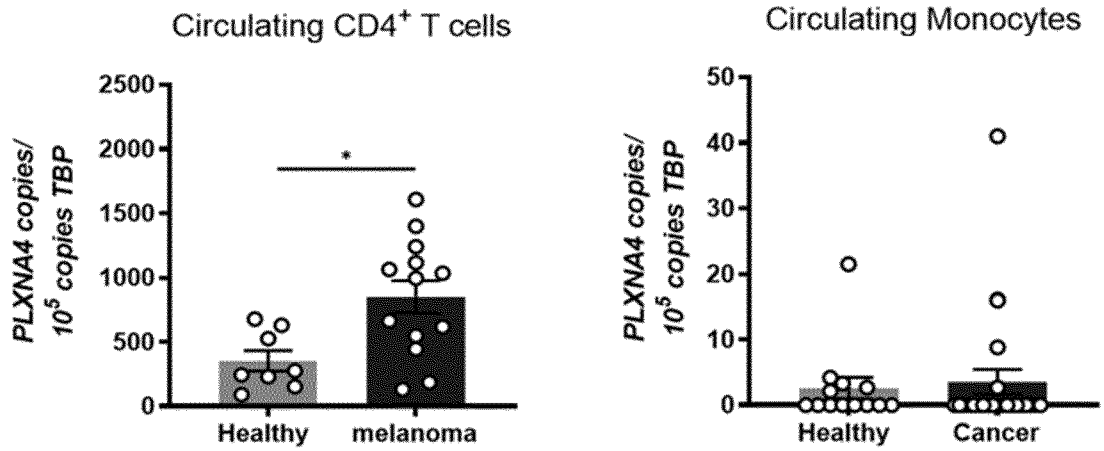

FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/076314

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6886 G01N33/574
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AI OLIVEIRA ET AL: "PlexinA4 plays a role in cancer progression and immune cell infiltration", POSTER PRESENTATION: TUMOUR BIOLOGY, June 2018 (2018-06), pages A344.1-A344, XP055668574, DOI: 10.1136/esmoopen-2018-EACR25.810 abstract ----- WO 2004/108896 A2 (UNIV ARKANSAS [US]) 16 December 2004 (2004-12-16) claims 1-2 ----- -/--	1-15 1,3-15

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2021	12/01/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Eveleigh, Anna

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO XIANG-YANG ET AL: "PlexinA1 expression in gastric carcinoma and its relationship with tumor angiogenesis and proliferation", WORLD JOURNAL OF GASTROENTEROLOGY, vol. 13, no. 48, 2007, page 6558, XP055782039, CN ISSN: 1007-9327, DOI: 10.3748/wjg.v13.i48.6558 abstract page 6559, left-hand column, paragraph 2	1,3-15
X	WO 2018/137436 A1 (CHANGHAI HOSPITAL SHANGHAI [CN]) 2 August 2018 (2018-08-02) paragraph [0035]; claims 1-11	1,3-15
X	WO 2015/037009 A1 (PLEXICURE LTD [IL]) 19 March 2015 (2015-03-19) cited in the application page 42, paragraph 5 example 7 claims 1,60	1-15
X	WO 2019/030461 A1 (UNIV CLAUDE BERNARD LYON [FR]; CENTRE NAT RECH SCIENT [FR] ET AL.) 14 February 2019 (2019-02-14) example 4	1,3-15
X	WO 2015/049289 A2 (AIT AUSTRIAN INST TECHNOLOGY [AT]) 9 April 2015 (2015-04-09) paragraphs [0006], [0008]; claim 1	1,3-15
X	EP 2 417 457 A1 (VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETENSCHAPPELIJK ONDERZOEK) 15 February 2012 (2012-02-15) paragraphs [0061] - [0063]	1,3-15
X	WO 2019/046832 A1 (JUNO THERAPEUTICS INC [US]) 7 March 2019 (2019-03-07)	15
A	claims 4-5, 109, 133-134, 136, 138	1-14
X	US 2018/291459 A1 (AL-DEEN ASHAB HUSSAM [CA] ET AL) 11 October 2018 (2018-10-11)	15
A	paragraphs [0006], [0017] - [0018], [0023]; claims 1, 16, 18-21	1-14

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/076314

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/076314

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/076314

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